UNRAVELING THE SIGNALING PATHWAYS OF THE CD200 ACTIVATION RECEPTOR FAMILY AND THEIR IMPLICATIONS IN REGULATING ANTITUMOR RESPONSE IN GLIOBLASTOMA

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Dedication

To my loved Son, Santiago, and my adored Mom, Lucha, the bravest people I have ever met.

Abstract

Glioblastoma multiforme (GBM) is the most aggressive and incurable primary brain tumor with a current median overall survival of approximately 14 months. Immune checkpoint-based therapy has demonstrated successes in solid tumors including melanoma and lung cancer increasing overall survival, however, it has not been successful in combating Central Nervous System (CNS) tumors. Our studies seek to establish a successful checkpoint inhibitor-based immunotherapy model for treating GBM, and our central hypothesis is, synthetic ligands modulate CD200 activation receptors (CD200ARs) overriding the inhibitory effect mediated by CD200 binding to CD200IR.

The CRISPR/Cas9 system was used to generate different murine raw264.7 macrophages (MØs) cell lines expressing different combinations or a single CD200 receptor. The resultant cell lines were stimulated with the synthetic ligand, and the effects of this binding were studied.

The main achievements of this research were to demonstrate that CD200ARs stimulated by synthetic peptide-binding couples with DAP10, and stimulates downstream activation of phosphatidylinositol 3-kinase, Vav1, cJUN, and ERK1/2. Second, CD200ARs form complexes (CD200ARs 2&3) to interact with the peptide ligands to optimize the biological function of macrophages. Third, the signals initiated by CD200ARs/DAP10 induce cytokine secretion and immune activation that results in tumor control.

Our research reveals the signaling pathway of the CD200 immune checkpoint that leads to activation rather than suppression of immune cells and improves the response of GBM to vaccine-based immunotherapy.

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Introduction

1.1 General Aspects of Glioblastoma

1.1.1 Glioblastoma Epidemiology

Glioblastoma (GBM) is the most common and aggressive malignant primary brain tumor. GBM has the poorest overall survival with a < 5% 5-year survival time and median survival time of 14-15 months despite standard of care therapy, making this disease an important public health issue. (Aldape et al. 2003; ICBTRotUS 2012; Cagney and Alexander 2017). GBM accounts for 45.2% of malignant primary brain and CNS tumors, 54% of all gliomas, and 16% of all primary brain and CNS tumors (Tran and Rosenthal 2010). Treatment is multimodal, with safe surgical resection followed by radiation therapy (RT) and concurrent Temozolomide (TMZ) chemotherapy (Stupp et al. 2005; Stupp et al. 2009; Michaelsen et al. 2013). Moreover, the complex biology that GBM has is much unknown despite decades of research making GBM difficult to treat, so it remains largely incurable (Dunn et al. 2012) that leads to an enormous individual and societal burden (Hanif et al. 2017; Dorte et al. 2016; Chen et al. 2018; Arvold et al 2014).

Age. GBM is an elderly disease, and it mostly diagnosed at 64 years. It is rare in children, and it is reported in children among 0 to 19 years olds, accounting for only approximately 3% of all brain and CNS tumors (Chakrabarti et al. 2005). Glioblastoma accounts for 50% of glioma in all age groups (Hanif et al. 2017), but the peak incidence is in patients between 55 to 60 years old and a median age of 64 years (Xu et al. 2017; Schwartzbaum et al. 2006; Ostrom et al. 2013; Ostrom et al. 2014). In the United States,

primary malignant brain tumors are rare and account for about 2% of all adult cancers (American Cancer Society 2012), with an incidence for GBM of 3.19 per 100,000 people per year, and the age-adjusted GBM rates being 2.5 times higher in European Americans than in African Americans (Song et al. 2009). Overall, among brain tumors, GMB is the most common and most deadly in adults.

Gender. According to the WHO, the incidence of GBM is dependent on race and gender. The incidence in males is 1.6 times higher compared to females (3.97 vs. 2.53) (Wen and Kesari 2008; Ostrom et al. 2013). Primary GBM tumors occur most frequently in men (male-to-female ratio, 1:33) and secondary GBMs in women (male-to-female ratio, 0:65) (Ohgaki et al. 2004; Sturm et al. 2014).

Ethnicity. Patient long-term survival may be affected by Ethnicity. Blacks, Asian/Pacific Islanders (API), and American Indian/Alaska Native have a lower GBM incidence rate (IR) compare to whites who have the highest IR while Hispanics have better survival than non-Hispanics (Thakkar et al. 2014). Whites had 2 times higher IR as compared with blacks with lower incidence in Asian-Pacific Islanders, and American Indians (Ohgaki and Kleihues 2005; Wrensch et al. 2002; Patel et al. 2019).

1.1.2 Standard Care of Glioblastoma

Once a patient is diagnosed with GBM, when possible, surgical resection remains the first step followed by adjuvant radiotherapy and chemotherapy (Ryu et al. 2014; Huang et al. 2017). Since 2005, chemotherapy both during and following radiation

therapy is part of the first-line treatment for GBM. Temozolomide, an alkylating cytotoxic agent, is administered orally on a daily basis at a dose of 75 mg/m^2 throughout radiotherapy, usually 60 Gy, 2 Gy in 30 fractions. Four weeks later, magnetic resonance imaging (MRI) is repeated and TMZ is then given at a dose of 150-200 mg/m^2 daily for 5 days in 28-day cycles for maintenance. A brain MRI is performed after every 2-3 cycles of TMZ treatment (or every 2-3 months) to ensure continued stable disease or response of the tumor to treatment (Stupp et al. 2009).

According to the results of the phase III EORTC 26981, TMZ treatment along with radiotherapy resulted in an improved median overall survival (OS) from 12.1 to 14.6 months, and an increase in the 2-year survival rate from 10% to 27% (Stupp et al. 2005; Mirimanoff and Stupp 2007). The cytotoxic activity and apoptosis caused by TMZ and other alkylating agents are apparent by the formation of O-6-methylguanine DNA adducts, which are repaired by the enzyme O-6-methylguanine-DNA methyltransferase (MGMT) (Quinn et al. 2009). Methylation of the MGMT promoter in tumor cells results in decreased expression of this enzyme and thus renders tumor cells more susceptible to alkylating agents (Hegi et al. 2005). Likewise, the primary mechanism of resistance to TMZ is dependent on MGMT activity (Wick 2009). High levels of MGMT activity in cancer cells create a resistant phenotype by blunting the therapeutic effect of alkylating agents and may be an important determinant of treatment failure (St-Coeur et al. 2015). Thus, novel approaches to treat glioblastoma remain a great need, since all current therapeutics (whether surgery, chemotherapy and/or radiation) fail to cure GBM. However, any new therapy should be specific and controllable and should also be able to

cross the BBB to be effective. Peptide-derived vaccines, which are an immunotherapy strategy might be the next-generation therapy to treat cancer.

1.2 Immunology of Glioblastoma

1.2.1. Introduction

Cancer immunotherapy has become a promising therapeutic modality to treat many tumor types, including GBM, but cancer cells have developed multiples mechanisms to evade the effector functions of the immune system and hinder the efficacy of effective immunotherapy, eventually. (Gonzalez et al. 2018; Jackson et al. 2019) . In GBM, for example, tumor heterogeneity is a hallmark. There are few targets and the same targets are not present in all patients. Hence, one drug may not be useful to treat most patients (Friedmann-Morvinski 2014). Another aspect to consider is, the blood-brain barrier (BBB) that poses several challenges for effective drug delivery (Harder et al. 2018). One challenge is, reaching and maintaining effective CNS permeation and drug concentration. CNS tumors, including GBM, compromise the structural integrity of the BBB causing more permeability at the tumor core (Chack et al. 2013).

The suppressive microenvironment in GBM allows immune escape and is mediated by many mechanisms (McGranahan et al. 2016; Wieser et al. 2018; Lehtipuro et al. 2019; Zhai et al. 2015). These include but are not limited to: 1. secretion of immunosuppressive cytokines (Transforming growth factor beta (TGF-β) and Interleukin 10 (IL-10)), 2. depletion of essential nutrients (by indoleamine dioxygenase (IDO) and arginase-1), 3. expression of inhibitory molecules such as Fas ligand (FasL), programmed cell death protein (PD-1) and its associated ligand (PD-L1), cluster differentiation 200

(CD200), and 4. recruitment or induction of immunosuppressive cells, such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). (Wang et al. 2018; Zhai et al. 2015; Olin et al. 2014). Thus, these immunosuppressive mechanisms, in conjunction with its propensity to infiltrate vital brain structures, and the regenerative capacity of treatment-resistant cancer stem cells (Jackson et al. 2019), make GMB a disease difficult to treat. However, curative immunotherapy, therefore, must break GBM immunosuppression and induce immune activation.

1.2.2 Immunosuppression Mechanisms of Glioblastoma

Systemic immunosuppression is a hallmark in GBM patients, and these immunological mechanisms are incompletely defined, but seems to involve both tumor-intrinsic factors and host response to tumor antigens originating from CNS (Jackson et al. 2019). Tumor cell-intrinsic factors are mostly related to intertumoral and intratumoral heterogeneity (ITH) that shape the archetype of GBM, and these include not only random mutations but critical drivers of cell survival and proliferation. Tumor-intrinsic factors include downregulation of neoantigens, alterations in IFN-γ signaling, activation of WNT–β-catenin pathway, mutations in the PTEN11 gene and loss of heterozygosity of loci containing genes encoding human leukocyte antigens (Jackson et al. 2019; Sharma et al. 2017; Zhao et al. 2019). Signaling pathways and the expression of immune check point molecules like IDO, PD-1, arginase-1, TGF-β, and signal transducer and activator of transcription 3 (STAT3) that suppress an immune response, are tumor-extrinsic factors or host response involved in GBM-associated immunosuppression (Bloch et al. 2013;

Pardoll et al. 2012; Veglia et al. 2018). Thus, for these, intrinsic and extrinsic factors, GBM is classified as high intrinsic resistance and high adaptive resistance (Jackson et al 2019) making GBM a recalcitrant tumor.

GBM tumor-intrinsic factors. ITH is the baseline of why GBM exhibits transiently potent adaptive and acquired resistance mechanisms. For example, one study collected spatially distinct tumor fragments from 11 GB patients and discovered different GBM molecular subtypes are within the same tumor (Sottoriva et al. 2013). Moreover, it has found intratumor heterogeneity at the copy number level at different stages of cancer progression. Some malignant clones exhibited loss alteration in CDKN2A/B and amplification of EGFR, CDK6, and MET at early stages, and copy number aberrations in genomic regions containing PDGFRA, PTEN, and TP53 occur at the latter stages (McLendon et al. 2008; Verhaak et al. 2010; Sottoriva et al. 2013). Furthermore, ITH has demonstrated in recurrence of GBM. Another study showed that recurrent and initial GBM share around 50% of the mutations, only, suggesting that therapy selects few early-stage clones or drives bottleneck for tumor evolution at the very early stage (Johnson et al. 2014; Kim et al. 2015). Thus, molecular heterogeneity undoubtedly represents an important mechanism of intrinsic resistance in GBM (Qazi et al. 2017), hampering the efficacy of immunotherapy.

Extrinsic mechanisms. GBM cells induce T cell tolerance and immunosuppression through the expression of the enzyme, IDO, and leading to the activation, expansion, and recruitment of Treg (Wainwright et al. 2014). The role of STAT3 in suppressing immune cell activity in GBM has characterized by Ganguly et al. 2018. For instance, interleukin-2 (IL-2)-mediated STAT3 activity expands tumor-

associated Tregs enhancing the expression of Forkhead Box P3 protein (Foxp3) in CD4+CD25+ T cells (Kortylewski et al. 2005; See et al. 2015). STAT3 expression in antigen presenting cells (APC), such as tumor-associated macrophages or microglia, results in the suppression of anti-tumor mechanisms and tolerance to tumor antigens. STAT3 has been shown to skew effective T helper (Th) 1 responses toward suppressive Th17 responses (Kryczek et al. 2007). STAT3 expression is driven by IL-10 which, together with TGFβ and prostaglandins, are some of the immunosuppressive mediators identified in GBM patients (Ganguly et al. 2018).

Several studies have shown that immune escape mechanisms involve direct interactions between GBM cells and immune cells (Wang et al. 20018). Glioma cells, for instance, show high expression levels of non-classical major histocompatibility complex (MHC) class I proteins such as human leukocyte antigens (HLA) -E and -G (Friese et al. 2003; Gupta et al. 2013), which inhibit NK cell-mediated lysis most likely by interacting with killer cell immunoglobulin (Ig)-like receptors. (Wischhusen et al. 2005; Wiendl et al. 2002). GBM cells also express FasL inducing apoptosis in invading T cells via Fas/FasL interactions (Didenko et al. 2002) or triggering inhibitory T cell checkpoints by PD-L1 (Parsa et al. 2007; Avril et al. 2010).

Myeloid cells, particularly macrophages, are major contributors in GBM-associated immunosuppression, in addition to immunosuppressive molecules. (Wang et al. 20018). It has reported that glioma-associated macrophages express and secrete multiple factors including stress-inducible protein 1 (STI1), epidermal growth factor (EGF), TGF-β, and membrane-type 1 matrix metalloproteinase (MT1-MMP) to promote glioma cell survival, proliferation, and migration (Coniglio et al. 2012; Wesolowska et al.

2008). Glioma cells induce macrophage recruitment by releasing chemoattractants like the chemokine, C-X-C motif ligand 12 (CXCL12), glial cell-derived neurotrophic factor (GDNF), and colony stimulating factor 1 (CSF-1) (Sielska et al. 2013; Wang et al. 2012). Unlike other tumors such as melanoma or lung cancer, the paucity of T cells in the GBM tumor microenvironment is unique, and resident microglia and macrophages exceed infiltrating T cells (Dunn et al. 2007). In brief, GBM is a highly immunosuppressive tumor but the exact mechanism of immune escape is unknown.

1.2.3. Checkpoint Inhibitors

1.2.3.1 Cytotoxic T-lymphocyte Associated Protein 4 Inhibitors

CTLA-4 is a member of the B7 family and is constitutively expressed on Tregs and transiently upregulated on effector T cells (Brunet et al. 1987). CTLA-4 is a ligand for the co-stimulatory molecule CD28 on T cells, which is important for activation and proliferation of effector T cells. CTLA-4 binds to CD28 with a higher affinity than CD80 or CD86, so that CTLA-4 expressing T cells can outcompete CD28-expressing T cells for binding to these co-stimulatory molecules on APCs inhibiting T cell activation (Linsley et al. 1994; Pardoll et al. 2012).

The effectiveness of CTLA-4 inhibition has been proven in preclinical murine models of GBM. Mice bearing tumors induced with the murine orthotopic glioma cell line, SMA-560, were treated with CTLA-4 antibody and 80% of treated mice exhibited benefit in survival compared to 0% in the control group (Fecci et al. 2007). In another report, the same survival benefit was observed in mice treated with anti-CTLA-4 therapy after being challenged with GL26, a murine glioma cell line (Wainwright et al. 2014).

In humans, anti-CTLA-4 therapy, commercially known as ipilimumab, has been used to treat metastatic melanoma. In a cohort of melanoma patients, ipilimumab demonstrated a statistically significant 2.1month improvement in overall survival (Callahan 2010), and the majority of patients were alive after 2 years of being treated (Robert et al. 2011). Moreover, the benefit of survival was similar in both patients with brain metastases and patients without brain metastases, supporting the hypothesis of immunotherapy activity against CNS malignancies (Margoli et al. 2012). However, the use of CTLA-4 antibody has failed as a monotherapy to treat other cancer types (Chae et al. 2018) including CNS tumors and has been replaced by PD-1/PD-L1 therapy, or in with CTLA-4 which has demonstrated enhanced and broader clinical efficacy in various types of tumors, including GBM (Spencer et al. 2019). Hence, the combination of PD-1 and CTLA-4 monoclonal antibodies has been successful in increasing the response rates and median survival time in multiples cancer types and in cancer patients.

1.2.3.2 Programmed Cell Death Protein 1 and Programmed Death-Ligand Axis (PD-1/PD-L1 Axis)

PD-1 is expressed on activated T cells, B cells, and NK cells. (Ishida, et al. 1992), including tumor-specific T cells (Gros et al. 2014) and Tregs (Loise et al. 2009). Normal tissue expresses PD-L1 and, similar to CTLA-4, plays an important role in maintaining peripheral tolerance. PD-L1 is involved in controlling host immune responses and its expression is induced through exposure to IFN-γ (Han et al. 2009). Also, PD-L1 is expressed on immunosuppressive immune cells including macrophages, MDSCs, and Tregs, suggesting that PD-1/PD-L1 axis might be another mechanism of suppression in

tumor-infiltrating leukocytes (Pardoll 2012). The exact mechanism of how the PD-1/PD-L1 axis induces tumor immunosuppression is not entirely understood and might be multifactorial.

PD1 signaling has different outcomes anergic state, for example, results in the engagement of PD-1 on activated T cells, which makes them unable to proliferate or produce effector molecules upon engagement of the TCR cognate antigen. (Barber et al. 2006). On the other hand, the expression of PDL-1 by tumor cells results in resistance to the elimination of cancer cells by tumor-specific T cells (Dong et al. 2002). Additionally, an increased number of glioma cells expressing PD-L1 and higher levels of PD-L1 expression have been positively correlated with higher-grade gliomas (Garber et al. 2016).

In preclinical mouse models, disruption of the PD-1/PD-L1 pathway through the use of inhibitory monoclonal antibodies resulted in enhanced efficacy, suggesting that it might be a more dominant mechanism of immune evasion. For example, several studies showed improved overall survival with anti-PD1 or anti-PD-L1 antibodies compared to anti-CTLA-4 in mice with intracranially implanted GL261 (Wainwright et al. 2014; Zeng et al. 2013). Furthermore, several clinical studies have shown that the combination therapy of the PD-1/ PD-L1, and CTLA-4 blockade is superior to either monotherapy, suggesting a nonredundant role of these immune checkpoint pathways in immune evasion.

Clinically, similar results have shown in preclinical models that combination immunotherapy, a treatment modality that combines two or more therapeutic agents, is a cornerstone of cancer therapy (Callahan et al. 2016; Bayat et al. 2017). A study showed

that TIM-3, which is an alternative immune checkpoint, in combination with PD1 improved survival in a preclinical model, synergistically (Kim et al. 2017). The dual therapy TGIT- and PD1-blocking antibodies synergistically improved survival in a preclinical model, compared to control and monotherapy groups. Moreover, the combination therapy increased effector T cell function and downregulation of suppressive Tregs and tumor-infiltrating dendritic cells (TIDCs) (Hung et al. 2018). Combination immunotherapy with anti-PD-1 and anti-CXCR4 in a murine glioma model significantly increased survival and improved CD4+/CD8+ ratios in the brain, contributing to increased levels of pro-inflammatory cytokines (Wu et al. 2019). Thus, combination therapy is effective in increasing the response and survival rates in preclinical mouse glioma models.

1.2.3.3 Toxicity Associated with Immune Checkpoint Therapy

Immunotherapy may be a new avenue to improve GBM treatment given its success in mouse glioma models and other tumor types. However, the increased use of immune-based therapies results in immune-related adverse events (irAEs). These immune-related adverse events result from non-specific activation of the immune system and can affect almost any organ (Eggermont et al. 2016). The incidence of any grade of irAEs has been reported to be as high as 90% with single-agent immune checkpoint inhibitor (ICI) therapy (Bertrand et al. 2015), but a meta-analysis of the genitourinary (GU) malignancies indicates an overall incidence of <75% with anti-CTLA-4 monotherapy (ipilimumab) and ≤30% in phase 3 trials of anti-PD-1/PD-L1 agents (Maughan et al. 2017; Topalian et al. 2012). IrAEs ≥ grade 3 severity occurred in up to

43% of patients taking ipilimumab and 20% taking PD-1/PD-L1 agents (Kumar et al. 2017). The incidence of irAEs is dose-dependent with ipilimumab and pembrolizumab, an anti-PD-1 antibody, showing greater toxicity at higher dose levels. Hence, toxicity also varies in the adjuvant and metastatic disease settings (Ascierto et al. 2017; Collins et al. 2017).

Dermatologic adverse effects more frequently related to the use of ICIs are maculopapular rash and pruritus, but lichenoid, eczematous, and bullous dermatitis, and psoriasis have also been reported. Dermatologic toxicity (all grades) was reported in 30–40% of patients taking PD-1/PD-L1 inhibitors and approximately 50% of patients treated with anti-CTLA-4 antibody, ipilimumab. Belum (Belum et al. 2016) reported that 13–20% of patients taking anti-PD-1 antibodies, pembrolizumab or nivolumab, developed rash or pruritus (all-grade) and approximately 8% of patients with melanoma developed vitiligo. Recently, hair re-pigmentation has also been described in patients treated with anti-PD-1 or anti-PD-L1 therapy (Rivera et al. 2017). Hence, ICI induces dermatologic affecting mainly skin.

Gastrointestinal adverse effects, such as diarrhea, are the most frequently reported irAEs in patients taking ICIs. The incidence of irAEs is higher (44%) in patients taking combination anti-CTLA-4/anti-PD-1 therapy than those receiving anti-CTLA-4 (23–33%) or anti-PD-1 (≤19%) monotherapy (Larkin et al. 2015). Another commonly reported irAE is hepatitis although it is less frequently observed. In patients treated with anti-PD-1 ICI, the incidence of hepatitis is approximately 5%, but in patients treated with combination ipilimumab and nivolumab this rises to 30% (Spain et al. 2016).

Pulmonary adverse effects, such as pneumonitis, are the most common lung toxicity observed in patients receiving ICI treatment. With monotherapy, the overall incidence of pneumonitis related to PD-1/PDL-1 and CTLA-4-targeted therapies was <5% and high-grade (≥ grade 3) irAEs occurring in 1–2% of patients. However, the incidence of pneumonitis with Combinations of PD-1 and CTLA-4 inhibitors was higher versus monotherapy (19 of 199 [10%] v 24 of 716 [3%]; P < .01) (Naidoo et al. 2017).

Infusion reactions are symptoms such as fever, rigor, pruritus, hypotension, dyspnea, chest discomfort, rash, urticaria, angioedema, wheezing or tachycardia that occur while the inhibitors are being administered. Infusion reactions were reported in 25% of patients receiving avelumab so premedication with acetaminophen and an antihistamine is recommended and in < 10% of patients receiving other approved immune checkpoints inhibitors (Genentech 2017).

1.3 Targeting the CD200 Immune Checkpoint

1.3.1 Introduction

CD200 is an immunosuppressive protein that belongs to the immunoglobulin superfamily. The CD200 protein is a widely expressed cell surface protein in a variety of tissues and cells including B cells, T cells, kidney cells, placenta cells, neurons and others (Wright et al. 2003). CD200 consists of extracellular, transmembrane, and intracellular domains. The intracellular region of CD200 protein lacks a signaling motif, therefore signaling by CD200 must be transduced via its receptors (Wright et al. 2000; Wright et al. 2003). In contrast, the CD200 receptor (CD200R) whose expression is more restricted to cells of the myeloid and lymphoid lineage has a long intracellular region with a

signaling motif that transmits signals affecting responses in multiple physiological systems. Thus, CD200/CD200R axis expression exerts effects on cancer growth, autoimmune and allergic disorders, infection, transplantation, bone development and homeostasis, and reproductive biology through the intracytoplasmic signaling motif of CD200R.

Several related genes coding for CD200 receptors in addition to that for CD200R have been identified. These genes have been called the activation receptors of CD200 protein because the encoding proteins lack cytoplasmic domains able to recruit signaling molecules directly and are thought to function by coopting accessory molecules (DAP10/12) for their function (Wright et al. 2003; Voehringer et al. 2004). However, whether these gene products also regulate immune function is controversial. Hence, understanding how the CD200 protein and its receptors modulate the immune system has implications in the development of new therapeutic approaches.

1.3.2 Distribution and Identification of CD200 Protein and Its Receptor CD200R

In 1982, the CD200 protein was initially discovered in rats and it was called OX2. It was characterized as a 41- to 47-kDa cell surface glycoprotein. In 2000, the OX2 protein was designated CD200 and will be referred to by this terminology for the remainder of this thesis. It is expressed on a variety of cell types, including thymocytes, B cells, activated T cells, follicular dendritic cells, endothelium (McMaster 1979), neurons in the central nervous system (including retina and optic nerve) (Webb 1984, Dick et al. 2001), cells of reproductive organs (Bukovský et al. 1984), and kidney glomeruli (Wright et al. 2001). It is thought the CD200 protein has a particular and important biological

function because of its conserved, unusual and specific, rather than ubiquitous, distribution. Sequence analysis revealed it belonged to the immunoglobulin superfamily (IgSF) and contained two IgSF domains, a single transmembrane domain and a short cytoplasmic domain. Evolutionary studies showed that CD200 distribution was relatively conserved across species consistent with the molecule having an important biological function (Barclay and Ward 1982).

Separately, two groups used recombinant DNA technology to identify a molecule (OX2R or CD200R) recognized by an antibody that blocked CD200 binding to macrophages. CD200R was purified from rat spleen cells and identified as a novel protein, similar in structure to CD200 that contained two IgSF domains, but with a larger cytoplasmic domain thereby having potential signaling capacity (Wright et al. 2000). Phenotypic analysis revealed the receptor was expressed by cells of myeloid lineage. Hence, the CD200/CD200R axis distribution has similarities with the CD47-CD172a Signal regulatory protein alpha (SIRP-α) interaction in that CD47 is widely distributed and its receptor, CD172a, is mostly expressed by myeloid cells. Signaling via CD172a has been shown to downregulate myeloid cells through tyrosine phosphatases, Src homology 2 domain-containing protein tyrosine phosphatase 1(SHP1) and Src homology region 2-containing protein tyrosine phosphatase 2 (SHP2) (Kharitonenkov et al. 1994).

Basic studies into the role of the CD200 protein in biological systems have raised tantalizing possibilities about the function of CD200 in the immune system. One hypothesis is that the CD200 protein is a costimulatory molecule, which is supported by the following facts, first, the gene-encoding for the CD200 protein and the costimulatory molecules, CD80 and CD86, are closely linked on chromosome 3 in mouse model

(Borriello et al. 1998), 2) CD80 and CD86 on APC are the ligands for the activating receptor CD28 and inhibitory receptor CD152 which are expressed on T cells (Linsley et al. 1993; Beyersdorf et al. 2015), 3) CD80 and CD86 are structurally related to OX2, each having one IgV and one IgC-like domain since these molecules belong to Ig family protein (Linsley et al. 1994).T cells stimulation assay using CD200 CHO cell transfectants failed to generate IL-2, IL-4 or IFN-γ, and T-cell growth and immune activation associated cytokines, leading to the conclusion that co-stimulation was incomplete, and this response was independent of the CD28/CD152 pathway (Borriello et al. 1998). However, work confirming the role of CD200 as a costimulatory molecule has not reported until now.

The immunosuppressive role of CD200 protein has been demonstrated by several studies. Gorczynski reported increased graft survival in an *in vivo* mouse model of allo- and xenograft transplantation due to the expression of CD200 on a subpopulation of NLDC145+ dendritic cells. They suggested that the engagement between CD200 and its CD200R delivered an immunosuppressive signal to these dendritic cells. Additionally, the increased graft survival could be reversed using a monoclonal antibody (mAb) specific for mouse CD200 (Gorczynski et al. 1999). Thus, these studies show that the natural functions of CD200 protein remain controversial; in some contexts, CD200 exerts costimulatory functions, although can also seems to deliver immunosuppressive signals.

1.3.3 CD200 Receptor Family

1.3.3.1 The Inhibitory CD200 Receptor (CD200R)

The inhibitory receptor CD200R (from human, mouse rat) was originally described by Barclay and colleagues in Oxford, and there is a consensus that CD200 is its natural ligand. (Wright et al. 2003; Voehringer et al. 2004; Hatherley et al. 2005). The CD200R and CD200 are closely related molecules implying evolution from a common ancestral protein (Wright et al. 2000). The human CD200R gene was characterized and mapped to chromosome 3q12-13 (Vieites et al. 2003). The human and mouse the tissue distribution, chromosomal location, and sequence of CD200R was confirmed by Gorczynski and colleagues (Gorczynski et al. 2004). CD200R exhibits a high content of N-linked glycosylation sites, 8 in the rat and 10 in the mouse (Preston et al. 1997). In addition, the cytoplasmic region of the receptor contained a NPXY motif that can interact with a phosphotyrosine-binding (PTB) domain present in several signaling adaptor molecules. The binding affinity of the interaction between CD200 and CD200R is comparatively low (KD = 2.5 μM) typical of interactions between migratory cells (Van der Merwe et al. 1993).

Unlike other well described myeloid inhibitory immune receptors such as FcεRIIB, GP49B1, or paired Ig-like receptors (PIRs), CD200R does not contain an immunotyrosine-based inhibitory motif (ITIM) (Ravetch and Lanier 2000). Tyrosine phosphorylation occurring following CD200:CD200R interactions depends upon the NPXY motif, leading to phosphorylation of docking protein 1(Dok1) and docking protein 1(Dok2) proteins, binding of Rasfamily GTPase-activating proteins (Ras-GAP) and Src homology 2 domain-containing protein tyrosine phosphatase (SHIP), and subsequent

downstream inhibition of the Ras-MAPK pathways (Zhang et al. 2004). Mihrshahi and Brown (2010) reported that following CD200:CD200R interaction, the phosphorylation of Dok2 preceded phosphorylation of Dok1 and recruitment of different downstream proteins by Dok2 and Dok1. Dok1 recruited considerably less RasGAP than Dok2, which also recruited the adaptor molecule non-catalytic region of tyrosine kinase adaptor protein 1 (Nck). In contrast, phosphorylation of Dok1 led to recruitment of CrkL. Using knockdown of Dok1 and Crk-like protein (CrkL) expression in U937 cells, a human histiocytic lymphoma cells, they showed increased Dok2 phosphorylation and Ras-GAP recruitment to Dok2. The authors suggest a model in which Dok1 negatively regulates Dok2-mediated CD200R signaling through recruitment of CrkL (Mihrshahi and Brown 2010).

A series of 15-mer synthetic peptides designed on the CD200 sequence were characterized at the functional level. The ability of these molecules to block binding of CD200R to CD200 was studied using competitive ELISA and CD200Fc fusion protein. Results were also duplicated in a FACS-based assay using CD200Fc fusion protein and infusion of these peptides abrogated the protective effects of CD200Fc fusion proteins in a mouse allograft model indicating *in vivo* activity. The mechanisms of these peptides exert their effects needs to further be elucidated (Chen et al. 2005; Gorczynski et al. 2001).

1.3.3.2 CD200R Family Gene Products

At least four murine CD200R-related genes, termed mCD200RLa-c by Wright in 2003 but referred to here as CD200AR2, -AR3, -AR4, -AR5, and a single related human

gene designated hCD200RLa have been characterized (Wright et al. 2003). CD200La and Lb (CD200AR4 and CD200AR3) isoforms were characterized and showed close sequence homology to CD200R in the extracellular regions with short cytoplasmic regions containing a positively charged lysine residue in the transmembrane region. Immunoprecipitation confirmed that this lysine residue would form a salt bridge with DAP12 and enable signal transduction. The transmembrane region of hCD200RLa was also found to have a positively charged amino acid (Voehringer et al. 2004).

Wright GJ, et al. in 2003 reported the gene expression and tissue distribution of the CD200AR4 and AR3 using RT-PCR. The highest level of mCD200AR4 was observed in resting mast cells, but the activation via FcεR1 decreased. Furthermore, lower expression on Th2 cells was observed. In contrast, mCD200AR3 was primarily expressed in activated mast cells, polarized Th2 cells, and to a lesser degree in cultured DC, but was virtually undetectable in cultured macrophages. (Wright et al. 2003) These genes may have specific, rather than redundant functions in the murine immune response based on their differential gene expression (Wright et al. 2003; Hatherley et al. 2005). In contrast, the human CD200RLa amino acid analysis showed that it lacked two cysteine residues critical for expression. Today, these key points are assumed: 1) no CD200RL molecules bind CD200; 2) the human CD200RLa has not been expressed as a functional protein; 3) it is likely that these are activating receptors, and 4) the ligands and signaling pathways have not been identified.

The nomenclature in mice of the CD200 activation receptors was updated based on sequencing analysis by Gorczynski and colleagues in 2004. These were termed CD200AR2 (corresponding to CD200RLc), CD200AR3 (corresponding to CD200Lb),

and CD200AR4 (corresponding to CD200La). Comparison of sequence data from Wright in 2003 indicated NH2-terminal differences for CD200AR2 and AR3 compared with CD200RLc and CD200RLb. Charged residues in the cytoplasmic domains of CD200R2-4 were found, but whether they were associated with ITAM was not determined. Using flow cytometry, COS7 cells transfected with all CD200R isoforms appeared to bind FITC-labelled CD200Fc contradicting previous reports that only the inhibitory CD200R bound CD200. However, these experiments were conducted in the absence of DAP12 expression by the transfected cells and it is probable that positive binding observed in these studies was an artifact. Moreover, the co-expression of DAP-12 has been confirmed independently for CD200R3 (CD200RLb) (Voehringer et.al. 2004). These findings suggest that the CD200 receptor family resembles other gene/protein families such as the SIRPs, Ly49 natural killer cell, Ig-like receptors and PILR, which have both activating and inhibiting forms (Barclay et al. 2002; Long 1999; Seiffert et al. 2001).

Thus far, it has been shown that CD200 binds to the inhibitory CD200R. In 2005, Hatherley et al. demonstrated using flow cytometry that only cells expressing CD200R bound a CD200-Fc fusion protein. Using surface plasmon resonance, an optical technique that is utilized to measure of the receptor/ligand interaction was confirmed at the protein level that CD200R binds to CD200-Fc fusion protein. The affinity of CD200R binding was calculated as Kd ~ 4 μM. Binding of CD200AR4 was too weak to measure accurately and no binding of CD200AR3, CD200AR2, or CD200AR5 could be detected using this sensitive assay (Hatherley et al. 2005). Sequence analysis between CD200 and its receptors revealed that strain-specific gene CD200RLe appears to be the most closely related (91%) to CD200R compared with CD200AR2 (83%), CD200AR3 (39%), and

CD200AR4 (84%). Mutagenesis analysis showed that single residue change in the predicted β strands C and F of the human CD200 protein was sufficient to prevent binding (Hatherley and Barclay 2004). These findings are very important for potential therapies targeting the CD200/CD200R axis because it suggests that immune response can be modulated for the development of new cancer immunotherapy approaches.

1.3.4 The role of CD200 Protein in Immunosuppression

It has been shown that the CD200/CD200R axis has a role in maintaining immunological non-responsiveness in myeloid APCs (Taylor et al. 2005). However, CD200−/− mice do not develop spontaneous autoimmune disease implying that central tolerance is not compromised. Nevertheless, the role of the CD200 protein in reinforcing peripheral tolerance has not been tested. Rosenblum and colleagues (2004) discovered a novel mechanism where upregulation of CD200 reinforces suppression of immune reactivity to self-antigens under steady state conditions (Rosenblum et al. 2004). They concluded this because CD200 protein was increased on dendritic cells undergoing apoptosis *in vivo* as well as *in vitro*. Another mechanism by which CD200 protein reinforces peripheral tolerance may involve tryptophan metabolism by the enzyme, indoleamine 2,3-dioxygenase.

The ligation of CD200R using a CD200-Ig fusion protein induced IDO expression and function mimicking the effects of B7/CTLA4 signaling in plasmacytoid dendritic cells. These findings led to the hypothesis that the CD200/CD200R axis acts to reinforce the tolerogenic properties of certain DC subsets over the adjuvant activity of immunogenic subsets (Fallarino et al. 2004). This was then proved by showing that

CD200R-Ig signaling upregulated IDO via type-1 IFN induction, whereas CTLA-4-Ig induced IDO induction was IFN-γ-dependent, suggesting an additional or alternative route to IDO expression and regulation (Fallarino et al. 2004; Fallarino et al 2005)

1.3.5 The Significance of CD200:CD200R Axis in the Immune Response to Tumors

In 2001, infusion of soluble CD200Fc was used to attenuate tumor growth of murine transplantable thymoma (EL4) in C57BL/6 mice. This was the first indication that the CD200/CD200R axis may be relevant to cancer biology and, specifically, tumor immunity. Since 2001, several groups have reported upregulation and expression of CD200 protein in tumors. The CD200 protein is overexpressed in several cancer cell lines and/or tissues, including ovarian, melanoma, head and neck carcinoma, testicular, malignant mesothelioma, neuroblastoma, renal cell carcinoma, chronic lymphocytic leukemia, prostate, and breast and colon cancers (Siva et al. 2007; Kawasaki et al. 2007).

Fascinatingly, CD200 is implicated as a prognostic factor for several tumor types like multiple myeloma (MM), acute myeloid leukemia (AML), and brain tumors (Tiribelli et al. 2017; Falay et al. 2018). In MM and AML, CD200 was expressed in 78% and 43% of the patients, respectively, and a significant correlation was found between the levels of CD200 expression and overall survival (Moreaux et al. 2006; Tonks et al. 2007). For brain tumors, GBM cells expressed higher levels of CD200 compared to other types of brain tumors and high levels of soluble CD200 (sCD200) in serum were associated with poor prognosis (Moertel et al. 2014).

In human breast cancer, levels of sCD200 in patient serum samples correlated with aggressive disease and metastasis. Furthermore, increased levels of sCD200 in

chronic lymphocytic leukemia (CLL) patients were reported and these levels correlated with the aggressiveness of the disease (Wong et al. 2012). Moreover, CD200 is associated with cancer progression (i.e. metastasis or relapse) in cancers of the bladder, lung, breast, and prostate, as well as, melanoma and chronic myeloid leukemia (CML) (Moreaux et al. 2008).

These findings indicate that monitoring sCD200 as a biomarker may provide significant diagnostic and prognostic information in different cancers and that therapies directed at interrupting CD200/CD200R axis interactions may prove to have clinical relevance.

Chapter 2

Tumor-derived Vaccines Containing CD200 Inhibit Immune activation: Implications for Immunotherapy

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2.1 Abstract

There are over 400 ongoing clinical trials using tumor-derived vaccines. This approach is especially attractive for many types of brain tumors, including glioblastoma, yet so far, the clinical response is highly variable. One contributor to poor response is CD200, which acts as a checkpoint blockade, inducing immune tolerance. We demonstrate that, in response to vaccination, glioma-derived CD200 suppresses the anti-tumor immune response. In contrast, a CD200 peptide inhibitor that activates antigen -presenting cells overcomes immune tolerance. The addition of the CD200 inhibitor significantly increased leukocyte infiltration into the vaccine site, cytokine and chemokine production, and cytolytic activity. Our data therefore suggest that CD200 suppresses the immune system's response to vaccines, and that blocking CD200 could improve the efficacy of cancer immunotherapy.

2.2 Introduction

Despite over four decades of intense research into vaccine-based strategies for fighting cancer, the majority of immunotherapies against solid tumors still fail to achieve beneficial outcomes. This is especially true for the central nervous system (CNS) tumor

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glioblastoma multiforme (GBM). A recent search on ClinicalTrials.gov revealed over 400 open clinical trials using tumor cells as a source of antigens to stimulate an anti-tumor response; twenty-five of these are directed towards CNS tumors.

The use of tumors as a source of tumor-associated antigens clearly has advantages (Olin et al. 2014); however, most cancers have robust mechanisms for evading the immune system (Olin et al. 2014). Immune checkpoint inhibitory ligands and their receptors tightly control T-cell activation, maintaining self-tolerance and limiting immune-mediated collateral tissue damage. Checkpoint blockades such as Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 protein (PD-1) (Fife et al. 2008) have been targeted in multiple clinical trials, demonstrating some success (Olin et al. 2014) (Wang et al. 2015). We have extensively studied another checkpoint blockade (CD200/CD200R) responsible for shutting down the immune system (Moertel et al. 2014; Ramsay et al. 2014) making the CD200 blockade interaction an important target for cancer immunotherapy (Moreaux et al. 2008; Gorczynski et al. 2008; Kretz-Rommel et al. 2008).

CD200 has been well characterized as an immunosuppressive protein that inhibits immune responses through its receptor (Liao et al. 2013; Gorczynski et al. 2013; Kretz-Rommel et al. 2007). In healthy individuals, CD200 is distributed on a wide variety of tissues, including B-cells, activated T-cells, certain vascular endothelia, kidney, placenta cells, and neurons (Wright et al. 2001). In contrast to the distribution of CD200 ligand, its receptor, CD200 receptor (CD200R), is mainly expressed on myeloid cells (monocytes, granulocytes, dendritic cells). CD200R is also expressed on T-cells and B-cells, inactivating leukocytes through negative immune signals (Wright et al. 2003; Rijkers et

al. 2008; Hoek et al. 2000). High expression of CD200R has also been detected on differentiated central and effector memory T-cells. CD200R expression is particularly apparent in polarized Th2 cells (Caserta et al. 2012), resulting in the expansion of regulatory T-cells (Gorczynski et al. 2005; Curiel et al. 2004; Jenmalm et al. 2016).

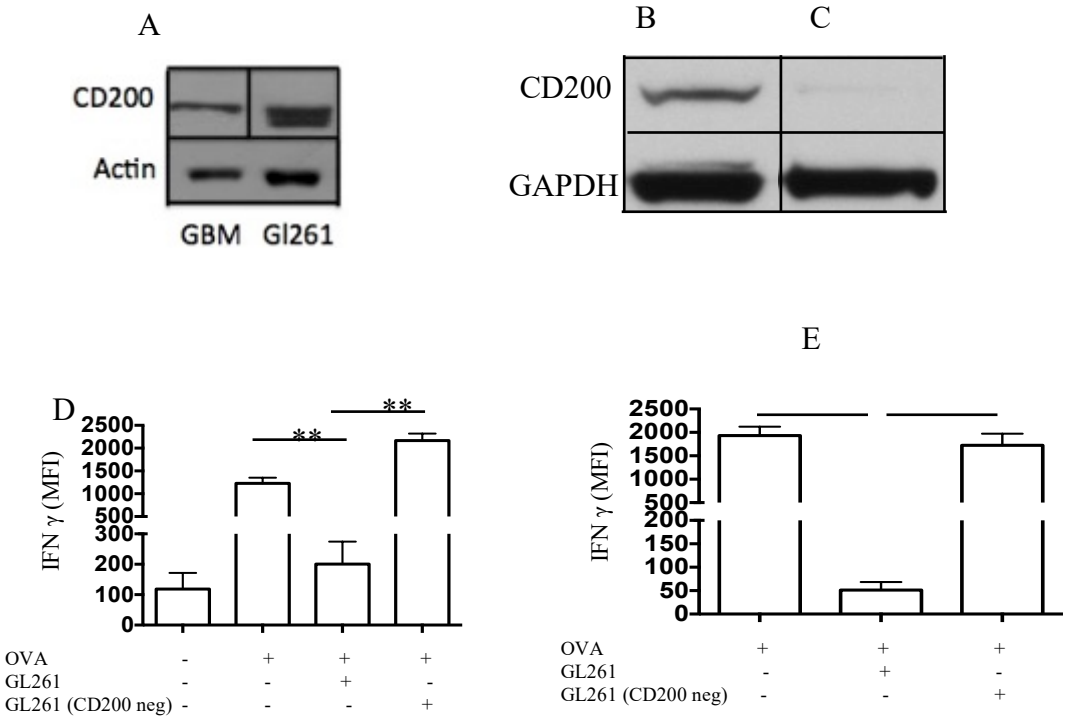
CD200 is expressed on tumors such as chronic lymphocytic leukemia (Kretz-Rommel et al. 2007) multiple myeloma (Moreaux et al. 2008), acute myeloid leukemia (Tonks et al. 2007), melanoma (Petermann et al. 2007), ovarian cancer (Siva et al. 2008), metastatic small cell carcinoma (Stumpfova et al. 2010), glioblastoma multiforme (GBM) (Moertel et al. 2014) and on the murine glioma GL261 (Figure 1A). In addition, tumor progression and poor patient outcome have been shown to correlate with the presence of soluble CD200 (Wong et al. 2012). Wong, et al. reported that soluble CD200 levels in the plasma of chronic lymphocytic leukemia patients correlate with tumor burden and disease state. In our phase I vaccine trial, we demonstrated increasing levels of CD200 in the serum of our GBM and ependymoma immunotherapy patients upon tumor recurrence (Moertel et al. 2014).

2.3 Results

2.3.1 Absorbing CD200 out of Tumor-derived Vaccines Enhances Immunogenicity

Because CD200 is expressed on tumors, we hypothesized that we are suppressing the immune system with the tumor-derived vaccines designed specifically to induce an anti-tumor immune response. To test our hypothesis, we depleted CD200 from our tumor lysates using immunoprecipitation (Figures 1B&C). OT-I splenocytes were pulsed with ovalbumin (OVA) + GL261 tumor lysate (GL261) or GL261 depleted of CD200 (GL261

(CD200neg)). GL261 significantly suppressed the ability of OVA to induce an immune response (p = 0.009), which was reverted by depleting CD200 from the vaccine (p = 0.003) (Figure 1D). Because CD200 acts on antigen-presenting cells (Jenmalm MC, et al. (2006), we repeated this experiment with bone marrow derived dendritic cells (DC). Our experiments recapitulated the findings in Figure 1D that, compared to OVA alone, tumor lysates containing CD200 inhibited IFN-γ (p = 0.001) and IL-2 (p = 0.005) production, a result which was reversed by depleting CD200 (p = 0.001) (IFN-γ) p = 0.0 (Figure1E) and (IL-2) (Figure 1F).



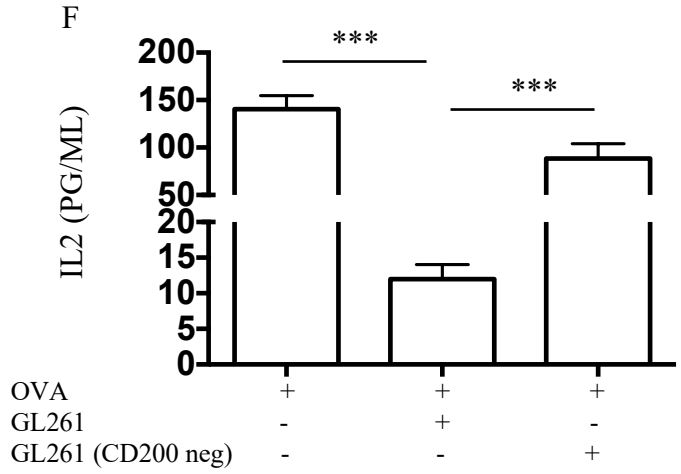
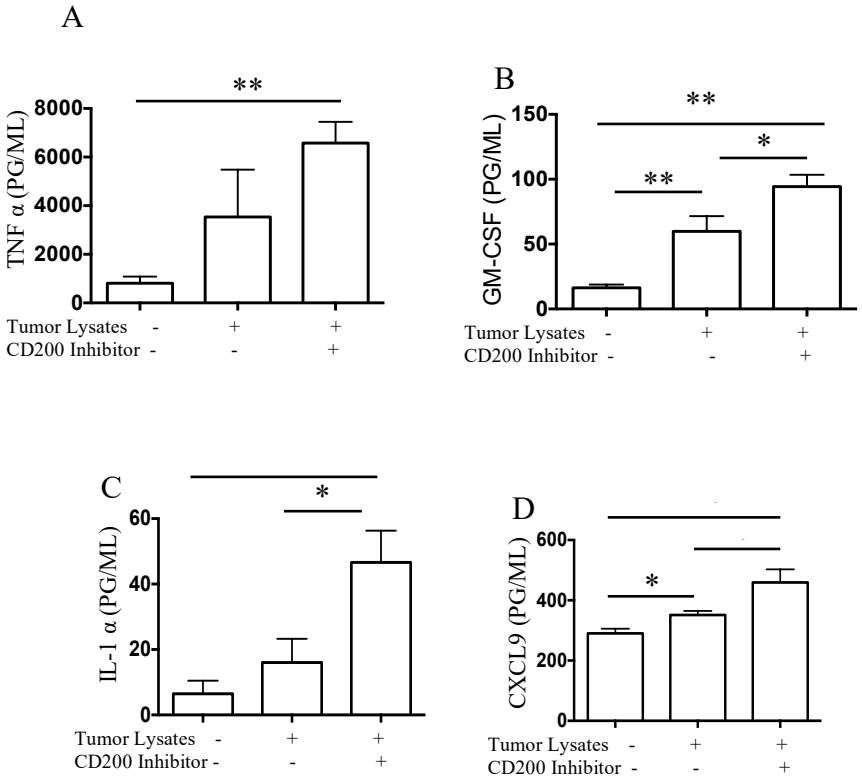


Figure 1. Absorbing CD200 out of Tumor-derived Vaccines Enhances Immunogenicity. (A) Human and mouse gliomas were analyzed by western analysis for CD200. (B&C) CD200 was absorbed out of murine GL261 tumor lysates and used to pulse (D) OT-1 splenocytes and (E) bone marrow derived dendritic cells with OVA as an immune stimulant with either wildtype GL261 or GL261 (CD200neg) tumor lysates. Error bars are ± SEM, asterisk represent a statistical significance * $p<0.05$, ** $p=0.005$ or *** $p=0.0005$ determined by unpaired t-test. Experiments are representative of three separate experiments.

2.3.2 CD200 Inhibitor Blocks Immune Suppression from Tumor-derived Vaccines

Targeting receptor-ligand interactions has become increasingly important, as indicated by CD200/CD200 receptor (CD200R) in leukemia cells and CD47/SIRP in many cancer cells (Akkaya et al. 2013; Gorczynski et al. 2005; Willingham et al. 2007). We developed a peptide inhibitor targeting the CD200R isoform activation receptors (Moertel et al. 2014). Purified CD11b cells from wildtype mice were pulsed with tumor lysate containing CD200, with or without the CD200 inhibitor. In these experiments, with the exception of TNF-α and IL1($p = 0.07$ and $p = 0.12$ respectively), tumor lysates alone elicited a statistically significant cytokine response ($p = 0.003$ (GM-CSF), $p = 0.012$

(IL6), p = 0.02 (CXCL9) and p = 0.006 (RANTES)) compared to no pulse controls. The CD200 inhibitor treatment group achieved a statistically significant enhanced immune response p = 0.004 ((TNF-α, p = 0.001 (GM-CSF), p = 0.033 (IL1b, p = 0.015 (CXCL9), p = 0.001 (IL6) and p = 0.013 (RANTES)) compared to no pulse control and (p = 0.015 (GM-CSF), p = 0.023 (IL1-α, p = 0.015 (CXCL9), p = 0.015 (IL6) and p = 0.046 (RANTES) compared to tumor lysate groups alone (Figures 2A-F). We observed enhanced secretion of TNF-α when adding the CD200 inhibitor to tumor lysates, however, these results failed to reach statistical significance (p = 0.069).



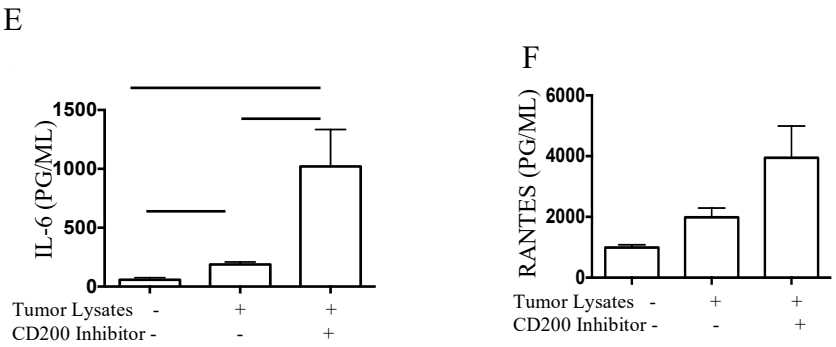


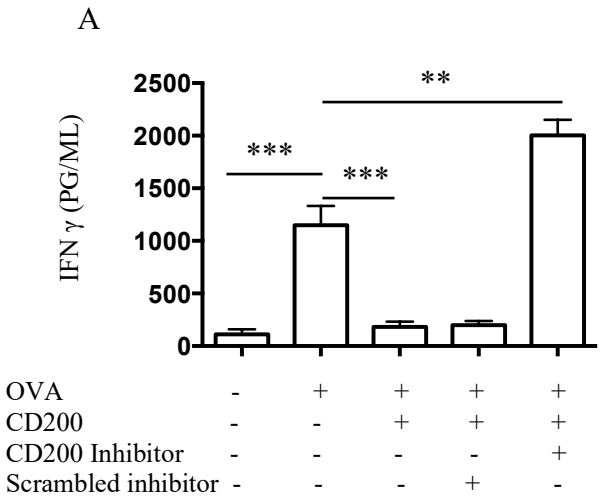
Figure 2. CD200 Peptide Inhibitor Blocks the Suppressive Properties of CD200. (A-F) CD11b isolated from C57BL/6 wildtype mice were pulsed with tumor lysates derived from wildtype Gl261 cells +/- the CD200 peptide inhibitor. Supernatants were analyzed for chemokine and cytokine secretion. Error bars are ± SEM, asterisk represent a statistical significance * $p<0.05$ or ** $p=0.005$ determined by unpaired t-test. Experiments are representative of three separate experiments.

2.3.3 CD200 Inhibitor Enhances an Antigen Specific Response

To generate a tumor-specific immune response, CD8 T-cells undergo priming by dendritic cells, the antigen-presenting cell most efficient at initiating potent CD8+ T-cell responses (Steinman et al. 1978; Banchereau et al. 1998). Currently, the efficacy of *ex-vivo* derived dendritic cell immunotherapy is not well established for human cancers (Soruri et al. 2005; Figdor et al. 2004; O'Neill et al. 2004; Olin et al. 2014). The limited success of these immunotherapies has been attributed to a variety of factors, including the preparation and administration of the vaccine, the disease stage of the participants in experimental trials, or the heterogeneous nature of most tumors. We suggest the failure to elicit an anti-tumor response is due to CD200 in tumor-derived vaccines used to activate dendritic cells.

To test this, bone marrow-derived dendritic cells (DC) from wildtype mice were pulsed with OVA + CD200 with or without the CD200 inhibitor. Following 24hr incubation, cells were washed to remove any free inhibitor, then incubated with purified OT-I cells. As previously demonstrated *in-vivo* (Moertel et al. 2014), the CD200 inhibitor

blocked the suppressive effects of CD200, reverting to an antigen-specific OVA immune response (Figure 3A). OVA significantly enhanced an IFN-γ response ($p = 0.007$), which was suppressed with the addition of CD200 ($p = 0.009$). The addition of the CD200 inhibitor overpowered the suppressive properties of the CD200 protein, significantly enhancing an immune response ($p = 0.003$), as compared to using OVA alone. Interestingly, in these experiments, we observed that cells pulsed with CD200 inhibitor + OVA, significantly enhanced the immune response ($p = 0.001$) (Figure 3B) compared to OVA treated cells. These studies led us to hypothesize that the CD200 inhibitor activates antigen-presenting cells.



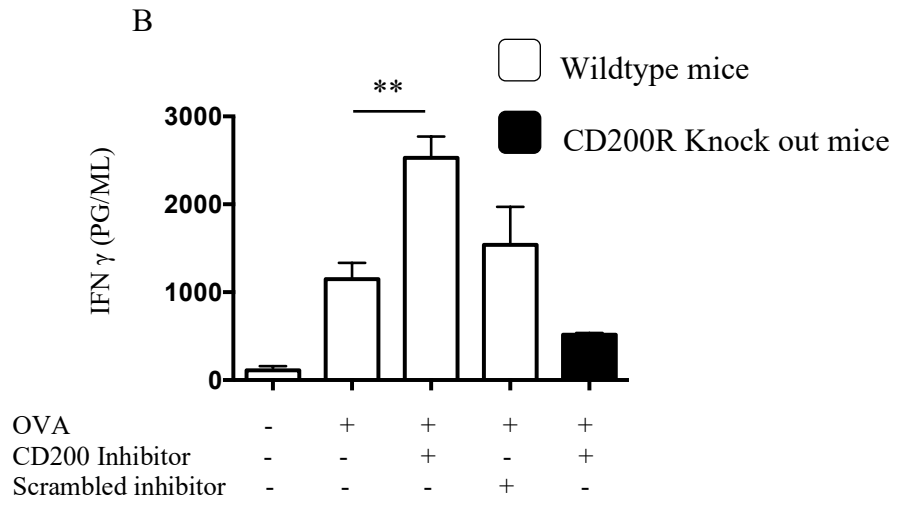


Figure 3. CD200 Inhibitor Enhances an Antigen Specific Response. (A & B) Bone-marrow derived dendritic cells from wildtype C57Bl/6 or CD200R KO mice were pulsed with OVA, OVA + CD200, OVA + CD200 + CD200 inhibitor or OVA + CD200 + scrambled inhibitor. Following 24hr incubation, cells were washed and purified and OT-I CD8 T-cells were added. Following 48hr incubation, supernatants were analyzed for IFN-γ production. Experiments are representative of three separate experiments. Error bars are ± SEM, asterisks represent a statistical significance * $p<0.05$, ** $p=0.005$ or *** $p=0.0005$ determined by unpaired t-test.

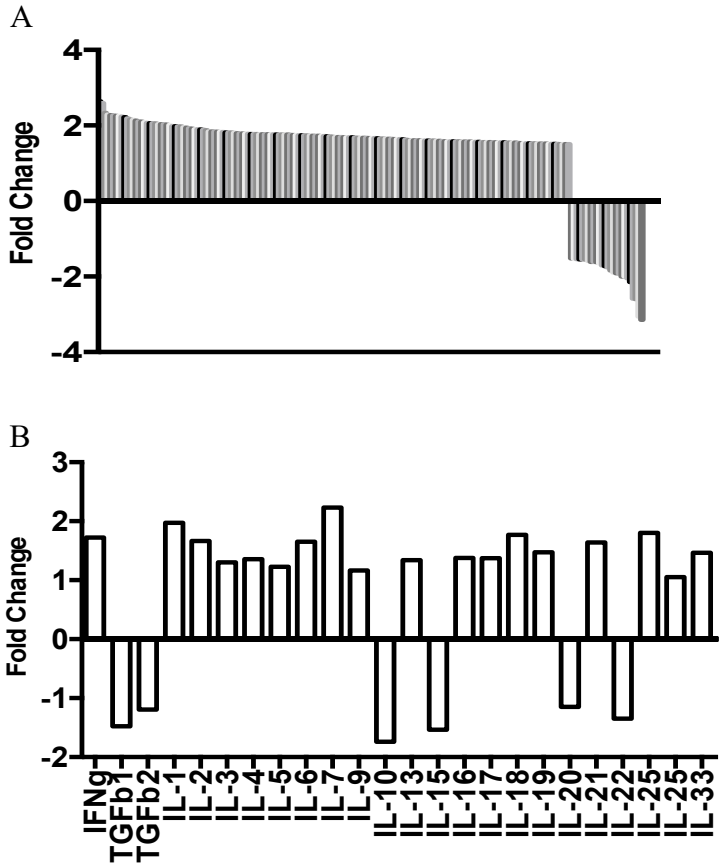
2.3.4 CD200 Inhibitor Modifies Gene Expression

To test our hypothesis that the CD200 inhibitor activates antigen-presenting cells, CD11b cells from wild type splenocytes were pulsed with CD200 protein, CD200 inhibitor, or a combination of CD200 protein + CD200 inhibitor and analyzed by NanoString for 575 immune related genes. All treatment groups were normalized to no pulse controls. In these experiments, 194 immune related genes had a +/- 1.5-fold change following pulsing with the CD200 inhibitor alone (Figures 4A-C).

When we compared all three treatment groups, we observed that ninety-eight genes within the CD200 protein group had an opposite response compared to genes within the CD200 inhibitor or CD200 protein + CD200 inhibitor treatment groups

(Figures 5A&B). These experiments demonstrated that the CD200 inhibitor reversed the inhibitory signaling induced by the CD200 protein.

To determine if the CD200 inhibitor activated functional responses within antigen-presenting cells, DCs were pulsed with the CD200 inhibitor alone. These experiments revealed that the CD200 inhibitor activated DCs, statistically enhancing the production of IL-2, TNFα, IL-1α, IL-6, GM-CSF, and IL-1α ($p = 0.01$, $p = 0.02$, $p = 0.04$, $p = 0.001$, $p = 0.004$, and $p=0.02$ respectively) (Figure 6A-H). We observed enhanced CXCL9 and IL-12 production, however, responses failed to reach statistical significance ($p = 0.055$ and $p = 0.32$, respectively).



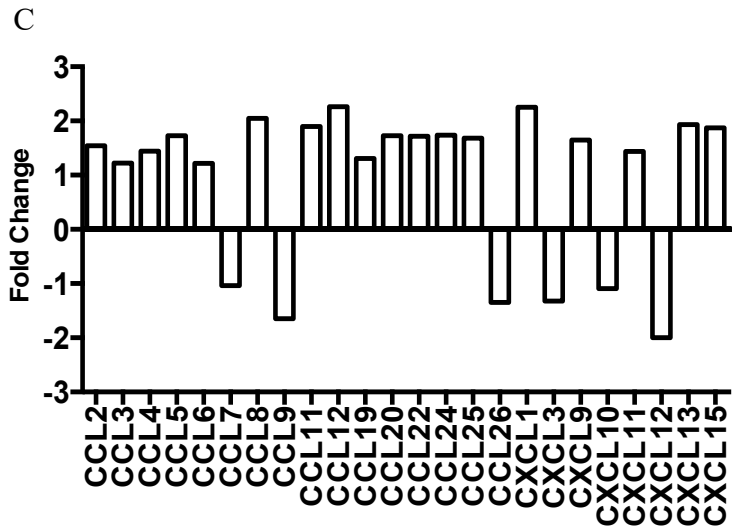
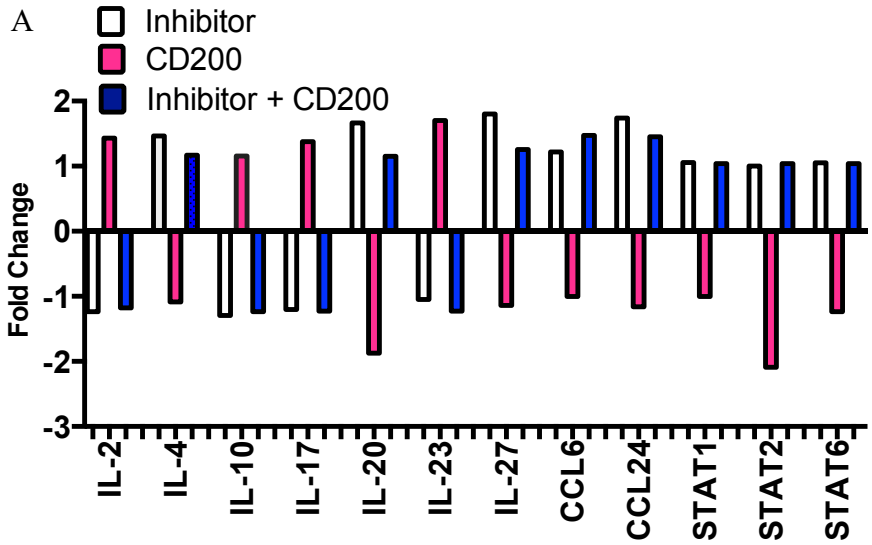


Figure 4. CD200 Inhibitor Modifies Gene Expression. Purified CD11b cells isolated from wildtype C5Bl/6 mice were pulsed with CD200 inhibitor. RNA was isolated and analyzed by NanoString for 575 immune related genes. Bars represent a (A) +/- 2-fold change or (B&C) +/- 1.5-fold change.



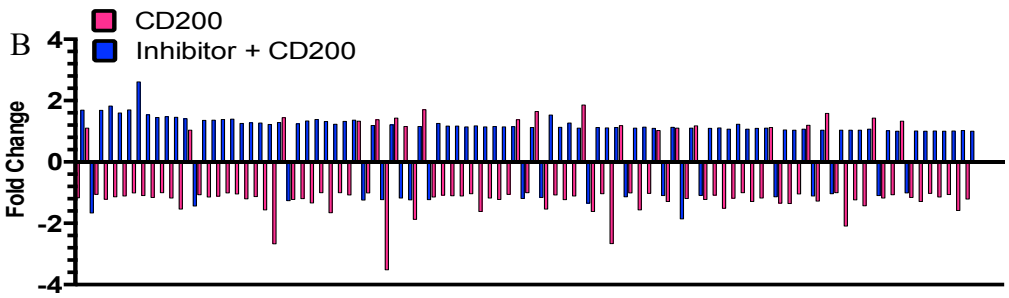


Figure 5. CD200 Inhibitor Reverses CD200 Protein Inhibitory Signals. Purified CD11b cells isolated from wildtype C5Bl/6 mice were pulsed with a CD200 protein, CD200 inhibitor or a combination of CD200 protein and CD200 inhibitor. RNA was isolated and analyzed by NanoString for 575 immune related genes. Bars represent a +/- 1.5-fold change.

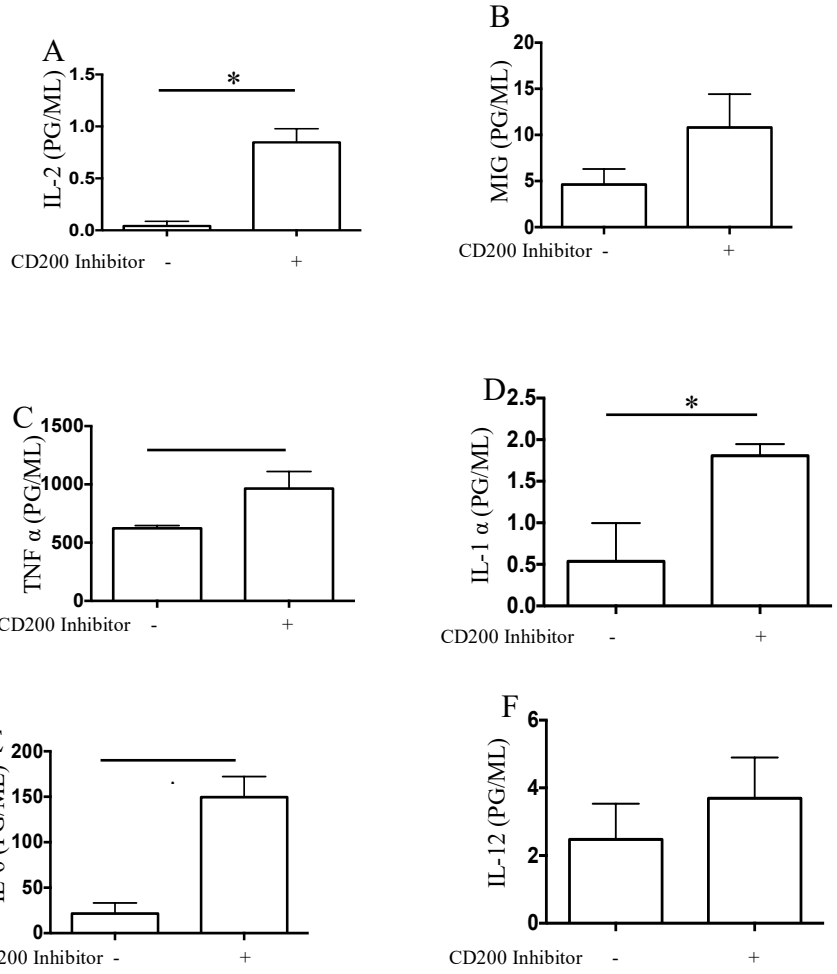


Figure 6. CD200 Inhibitor Stimulates Dendritic Cells. (A-H) Bone marrow derived dendritic cells from wildtype C57Bl/6 mice were pulsed with CD200 inhibitor. Following 48hr incubation, supernatants were analyzed for chemokine and cytokine production. Error bars are ± SEM, asterisk represent a statistical significance * $p<0.05$** or $p=0.005$ determined by unpaired t-test.

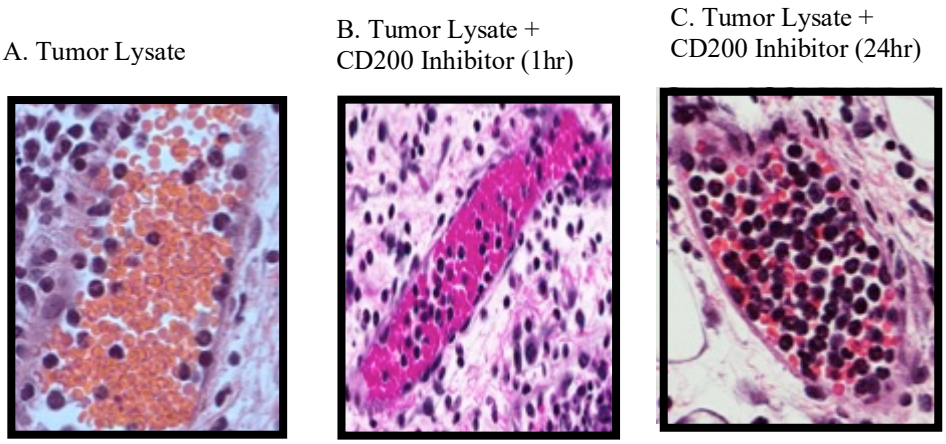
2.3.5 CD200 Inhibitor Enhances Leukocyte Trafficking into the Vaccine Site

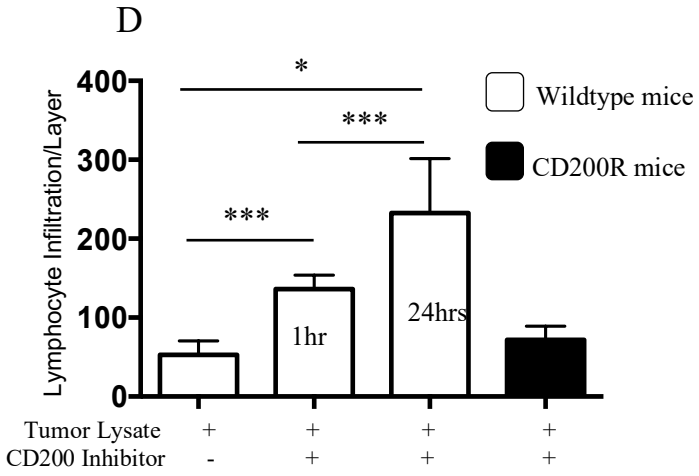
GM-CSF is often used in vaccines to enhance the infiltration of antigen-presenting cells into the vaccine site for antigen uptake and presentation (Morse et al. 2015). We found that the CD200 inhibitor enhanced production of GM-CSF (Figure 6G) *in vitro*. Therefore, in the next set of experiments, non-tumor-bearing wildtype and CD200R KO mice were vaccinated with tumor lysates or CD200 inhibitor alone. Twenty-four hours later, mice were revaccinated with tumor lysates + CpG-ODN or tumor lysates + CpG-ODN + CD200 inhibitor, respectively. In one of the treatment groups, mice were vaccinated with the CD200 inhibitor 1hr prior to revaccination with tumor lysates + CD200 inhibitor (Figure 7A-C).

Six hours following revaccination, skin at the vaccine site was harvested and analyzed for leukocyte infiltration. No significant leukocyte infiltration was observed in saline vaccinated controls or in CD200R KO mice vaccinated with tumor lysates + CD200 inhibitor (data not shown). To quantify our results, vascular leukocytes from eight layers of tissue were counted (Figure 7D). These experiments demonstrated enhanced leukocyte infiltration into the vaccine site with as little as 1hr pre-vaccination with the CD200 inhibitor ($p = 0.001$; 1hr and $p = 0.001$ 24hrs) (Figure 7D). Moreover, knocking out the CD200 receptor failed to enhance leukocyte infiltration ($p = 0.087$).

These experiments demonstrated that while we were capable of eliciting an immune response using tumor-derived vaccines, the response failed to recruit antigen-presenting cells to the site of vaccination for antigen uptake. We next wanted to see how removing CD200 from tumor lysate vaccines influenced leukocyte infiltration. In these

experiments, non-tumor-bearing wildtype mice were vaccinated with tumor lysate or tumor lysate void of CD200. Twenty-four hours later, mice were revaccinated with tumor lysate + CpG-ODN or tumor lysate void of CD200 + CpG-ODN respectively (Figure 7E). As seen in the above experiments, we observed a significant infiltration of leukocytes into the site of vaccination ($p = 0.004$), however, removal of CD200 profoundly enhanced leukocyte infiltration ($p = 0.0001$) (Figure 7E).





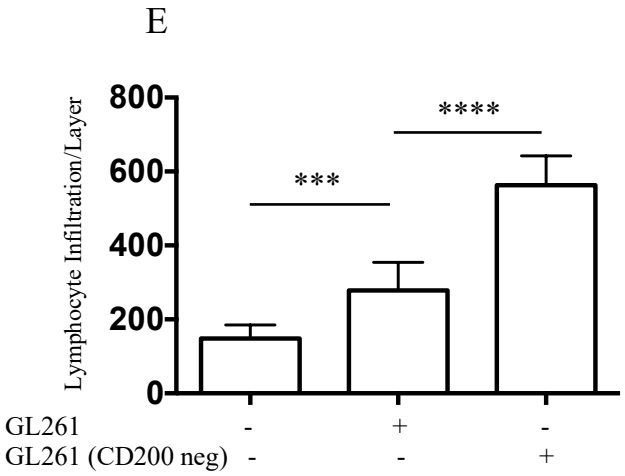


Figure 7. CD200 Inhibitor Enhances Leukocyte Trafficking into the Vaccine Site. Non-tumor bearing C57Bl/6 or CD200R knockout mice were vaccinated with tumor lysates or CD200 inhibitor, either 1hr or 24 hrs later, mice were revaccinated with (A) tumor lysates + CpG or (B) tumor lysates, CD200 inhibitor + CpG. (1hr revaccination) (C) tumor lysates, CD200 inhibitor + CpG. (24hrs revaccination). Six hours later, skin from the vaccine sites was harvested and analyzed by H&E staining. (D) Leukocytes within blood vessels in 8 separate skin levels were counted. (E) In separate experiments, mice were vaccinated with wildtype GL261 lysates or GL261 lysates void of CD200. Twenty-four hours later, mice were revaccinated with either wildtype GL261 lysates or GL261 lysates void of CD200 + CpG. Six hours later, skin was harvested and leukocytes within blood vessels in 8 separate skin levels were counted. Error bars are ± SEM, asterisks represent a statistical significance * $p<0.05$, ** $p=0.005$ and *** $p=0.0005$ determined by unpaired t-test.

2.3.6 CD200 Inhibitor Enhances an Anti-tumor Response

In the next set of experiments, we investigated the effects of CD200 inhibitor on the expression of co-stimulatory molecules. In these experiments, we used the OVA protein due to the SIINFEKL antigen's ability to stimulate an immune response. Purified CD11b cells isolated from wildtype mice were pulsed with OVA or OVA + CD200 inhibitor. Following 24hr incubation, CD200 inhibitor significantly enhanced CD80,

CD86, and MHC-II expression ($p = 0.012$, $p = 0.028$, and $p = 0.038$, respectively) as compared to no pulse controls (Figure 8A). CD200 inhibitor significantly enhanced the expression of CD80 and CD86 ($p = 0.032$ and 0.018, respectively) compared to the OVA alone treatment group.

To determine whether the use of our CD200 inhibitor would enhance functional responses, we used an in-vivo cytolytic model to investigate the effect of CD200 inhibitor on an anti-tumor response. In these experiments, wildtype or CD200R KO mice underwent intracranial inoculation as described by Olin et al. (Olin, et al. 2010). Mice were vaccinated with tumor lysates with or without the CD200 inhibitor (Figure 8B). Scrambled inhibitor was used as a control. Twenty days post-inoculation, lymphocytes from draining cervical lymph nodes were harvested and incubated with GL261 cells to initiate a tumoricidal response. Two-way ANOVA revealed a statistically significant enhancement of an anti-tumor response by lymphocytes with the addition of the CD200 inhibitor ($p = 0.001$) (Figure 8B).

Individual analysis by Student's T-test revealed that tumor lysates in both wildtype and CD200R knockout mice with significantly enhanced anti-tumor responses ($p = 0.001$ and $p = 0.001$) at effector:target cell ratios of 25:1 and 50:1, respectively, as compared to the saline treatment group. In addition, the CD200 inhibitor group significantly enhanced anti-tumor responses ($p = 0.001$, $p = 0.001$, and $p = 0.001$) at effector:target cell ratios of 5:1, 25:1, and 50:1, respectively, as compared to the saline treatment group. In wild type mice, the CD200 inhibitor treatment group exhibited significantly enhanced anti-tumor responses at effector:target cell ratios of 5:1, 25:1, and 50:1 ($p = 0.0001$, $p = 0.026$, and $p = 0.003$, respectively) as compared to the tumor lysate

treatment group. In addition, there were significantly enhanced anti-tumor responses between CD200 inhibitor and CD200 scrambled inhibitor control treatment groups in wildtype mice at effector:target cell ratios of 5:1, 25:1, and 50:1 ($p = 0.001$, $p = 0.0066$, and $p = 0.0018$, respectively). We also observed significantly enhanced anti-tumor responses at effector:target cell ratios 5:1, 25:1, and 50:1 ($p = 0.006$, $p = 0.016$, and $p = 0.006$, respectively) between wildtype and CD200R KO mice in the CD200 inhibitor treatment group. No significant differences were observed between wildtype and CD200R KO mice treated with tumor lysates or the scrambled inhibitor. These experiments demonstrated the ability of our inhibitor to attract leukocytes to the site of vaccination. This is important, researchers have often used GM-CSF with their vaccines to attract antigen-presenting cells the take up tumor specific antigens for presentation in the draining lymph nodes.

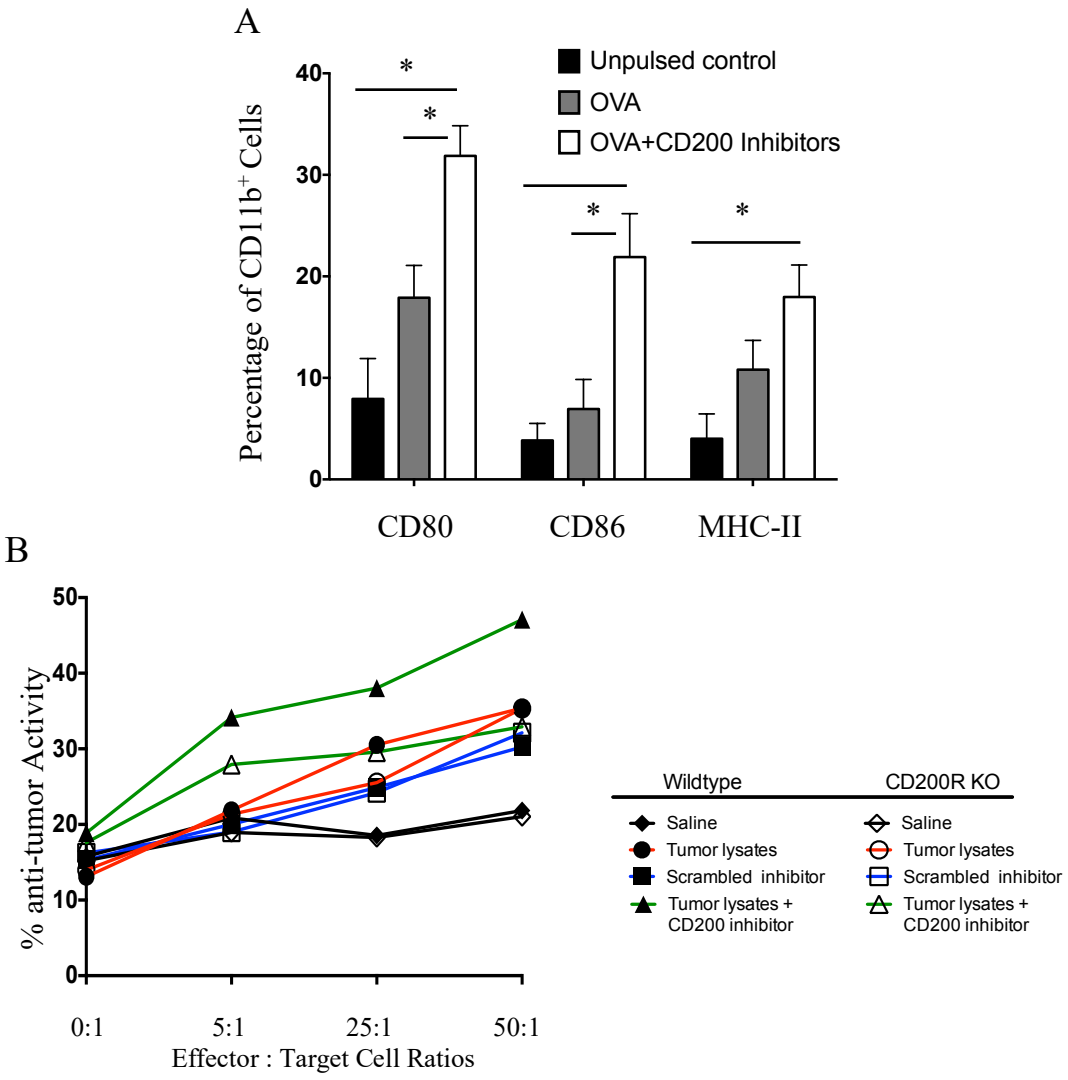


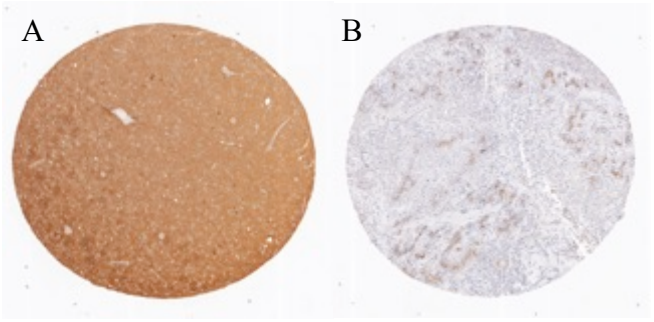
Figure 8. CD200 Inhibitor Enhances an Anti-tumor Response. (A) Purified CD11b cells from wildtype C57Bl/6 cells were pulsed with OVA +/- CD200 inhibitor. Forty-eight hours later, cells were analyzed for CD80/86 and MHC-II expression. (B) Tumor-bearing wildtype (solid symbols) or CD200 receptor knockout (CD200R KO) (open symbols) mice were vaccinated in the back of the neck with saline (black lines), wildtype GL261 tumor lysates (red lines), tumor lysates + scrambled CD200 inhibitor (blue lines) or tumor lysate + CD200 inhibitor (green lines). Twenty days post vaccination; lymphocytes from cervical lymph nodes were harvested, incubated for 6 hours with wildtype GL261 cells and analyzed for cytolytic activity. Asterisks represent statistical significance * $p<0.05$ determined by two-way ANOVA.

2.3.7 CD200 is Upregulated on Endothelial Cells

Inhibiting CD200/CD200R interactions has been suggested as a method to enhance immunotherapy (Gorczynski et al. 201; Gorczynski et al. 2013; Copland et al. 2007; Rygiel et al. 2012). A clinical trial sponsored by Alexin Pharmaceuticals (NCT00648739) developed a monoclonal anti-CD200 (ALXN6000) to block tumor-derived CD200 expressed on B-cell Chronic Lymphocytic Leukemia (B-CLL) and Multiple Myeloma cells from interacting with CD200R+ lymphocytes (clinicalTrials.gov) (Rygiel et al. 2012). No results have been posted in clinicaltrials.gov. We do not anticipate that this method will be a very efficacious therapy. Twito et al. has demonstrated that "A Disintegrin And Metalloprotease" enzyme (ADAM28) sheds CD200 from B-CLL (Twito et al. 2013), which would invalidate the use of an antibody to block tumor-driven CD200-CD200R interactions. Our preliminary data corroborates Twito's findings. We reported high transcription levels of CD200 in GBM (Moertel et al. 2014), however, staining for CD200 protein revealed that, in contrast to normal CNS, GBM have low CD200 expression (Figures 9A & B) potentially due to secretion.

To validate CD200 protein expression on GBM, human GBM for CD200 expression were analyzed by western analysis. In contrast to normal CNS tissue, there was low expression of CD200 on the tumors. However, closer examination revealed that GBMs increase expression of CD200 on endothelial cells within the blood brain barrier (Figure 9C). The same CD200 expression was seen in the vasculature of human breast carcinoma (Figure 9D) and melanoma (Figure 9E). To determine the ability of GBM to upregulates CD200, human endothelial cells (HUVEC) were placed on the bottom of a

trans-well plate and human GBM was placed on the top. Following 72hr incubation, HUVEC cells were harvested and analyzed by Western Immunoblot (Figure 9F) and RT-PCR (Figure 9G) for CD200. These experiments demonstrated that GBM induces CD200+ endothelial cells.



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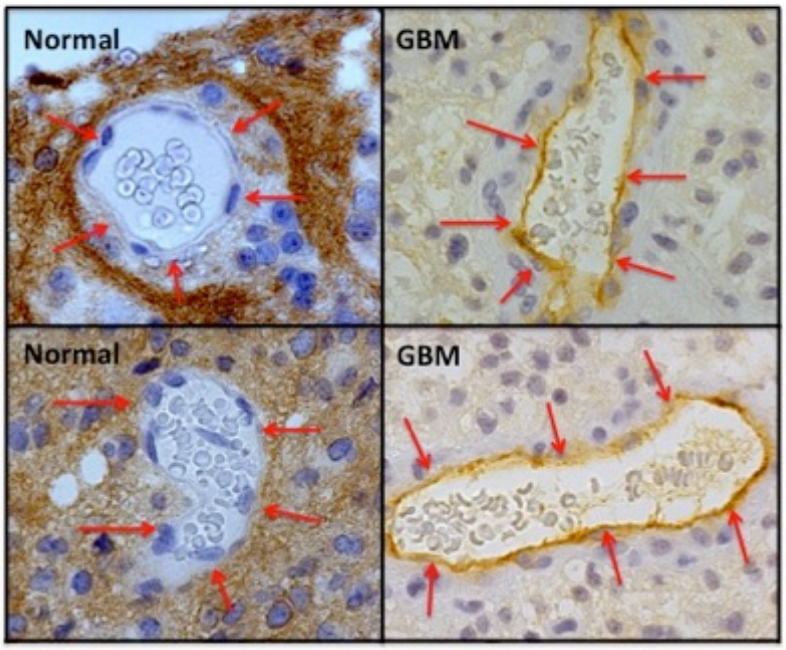

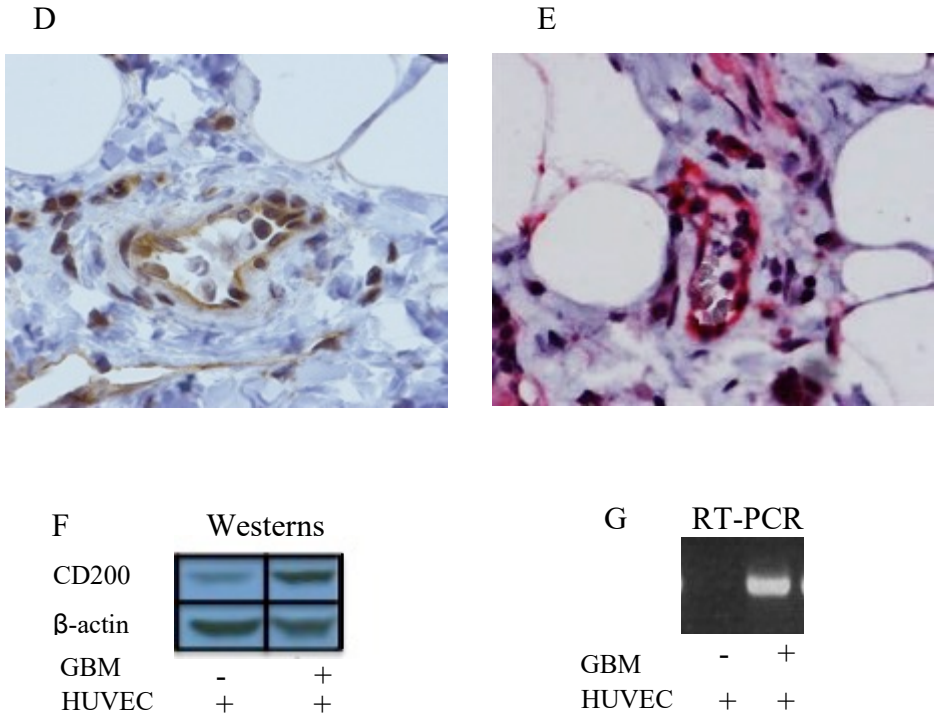

Figure 9. CD200 is Upregulated in Vascular Endothelial Cells. Tissues isolated from (A) normal human central nervous system or (B) glioblastoma multiforme were analyzed for CD200 expression. (C) Vascular endothelial cells from normal tissue and glioblastoma multiforme (GBM) (D) breast tumor and (E) melanoma cells were analyzed for CD200 expression. Human endothelial cells were expanded on the bottom of a trans-well plate. Glioblastoma multiforme cells were placed on the top of the plates and incubated for 48hrs. HUVEC were washed and analyzed by (F) western analysis and (G) RT-PCR for CD200 transcription.

2.4 Conclusions

CD200 has been well described as immunosuppressive, making it a logical target for immunotherapy (Moertel et al. 2014). We have been extensively interrogating the multiple mechanisms by which CD200 inhibits the development of an anti-tumor response. We suggest that the CD200 in tumor-derived vaccines and that the CD200 protein is secreted from the tumor microenvironment will inhibit the ability of antigen-presenting cells to mount an anti-tumor response (Figures 10A&B). We also argue that

our CD200 peptide inhibitor, through the activation of a CD200 isoform receptor, reverses CD200-induced suppression (Figure 10C).

Our model is supported by studies reporting that CD200/CD200R interactions have been characterized as inhibitory receptor (Gorczynski et al. 2004). CD200R contains tyrosine motifs which signal through the recruitment of Dok 2 to distinguish the CD200R from almost all other inhibitory receptors that have immunoreceptor tyrosine-based inhibition motifs. (Hatherley D, et al. 2013) However, additional CD200R-like proteins have recently been identified in mice and humans. (Wright et al. 2003) Four separate CD200 receptor genes have been identified: CD200R, CD200R2, CD200R3, and CD200R4 (Voehringer et al. 2004). These receptors are predicted to be associated with DNAX activating protein, (DAP12), known to potentiate and attenuate activation of leukocytes (Turnbull et al. 2007). Although the CD200R isoforms have not been well characterized, Gorczynski (2008) reported that specific peptide sequences within the CD200 protein act as antagonists. Gorczynski hypothesizes that these peptide sequences bind to one of the CD200R isoforms that normally contribute an activation signal (Gorczynski et al. 2008).

Our data correlates with Gorczynski's hypothesis. We suggest that our CD200 inhibitor is targeting one of the activation isoforms of the CD200 receptor. However, CD200 has multiple mechanisms of inducing immune suppression. Following close examination of CD200 immunohistochemistry, we have demonstrated that CD200 is upregulated on vascular endothelial cells (Figure 9C). CD200+ endothelial cells appear to be tumor-specific because the surrounding CNS does not express CD200 in the blood-brain barrier vasculature (data not shown). This is an important discovery because others

have reported that tumor-CD200 expression differentiates CD4+CD200R+ cells into a suppressor T-regulatory population (reviewed in Holmannova, et al. 2012). We suggest that CD200R-bearing leukocytes will interact with CD200+ endothelial cells to differentiate CD4+CD200R+ to regulatory T cells, leading to the development of an immunosuppressive tumor environment (Figure 10 C).

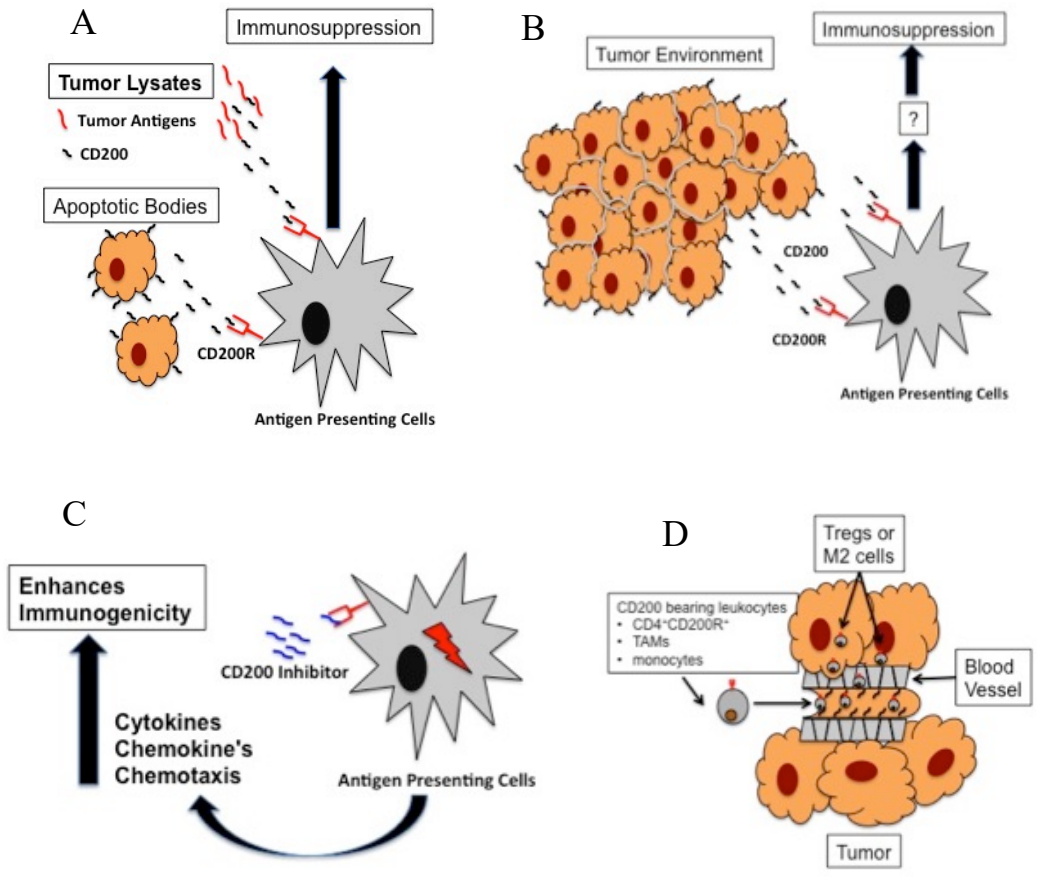

Figure 10. Experimental Models. (A) CD200 from tumor lysates or apoptotic bodies used for vaccines binds to the CD200R on antigen presenting cells within the vaccine site inhibiting the development of an antitumor response. (B) CD200 is solubilized from tumors binding to CD200 inhibitory receptors on antigen-presenting cells in the draining lymph nodes inhibiting the development of an immune response. (C) Experimental model demonstrating how the CD200

inhibitor binds to the CD200 isoform activation receptors, over-riding the inhibitory signals of the CD200 protein. (D) CD200+ endothelial cells within the vasculature bind to CD200R+ lymphocytes, differentiating them into suppressor cell populations.

2.5 Future Plans

Breaking CD200/CD200R interactions intensifies the success of antitumor therapy. We developed a 13 amino acid CD200 peptide inhibitor that, given with tumor lysate, significantly enhances immunogenicity in our glioma model, as well as our breast carcinoma model. We are now focusing our efforts on a mechanism to overcome the suppressive CD200+ endothelial cells (Figure 9C). We are developing a monoclonal anti-CD200R specific for the same epitope as our CD200 inhibitor, which we hope will block the differentiation of immune suppressor cells. We hypothesize that, following T-cell activation, systemic inoculation of the anti-CD200R will bind the CD200R on CD200R+ leukocytes. Our preliminary data suggests that blocking CD200R will allow CD200R leukocytes to enter the tumor microenvironment, escaping differentiation into their suppressive populations.

Financial & competing interests disclosure. The authors have no financial disclosures of competing interest.

Ethical conduct statement. All experiments in these studies were conducted within the Institutional review board and institutional animal care and use committee regulations.

2.6 Acknowledgments

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Chapter 3

Binding Analysis of the CD200 Activation Receptors

3.1 Introduction

While advances in cancer immunotherapy have occurred over the past two decades, the strategies developed by tumors to circumvent immune surveillance and tumor-mediated immunosuppression constitute a critical hurdle for the development of successful immunotherapies (Ribatti 2017). This is unquestionably true for malignant brain tumors such as glioblastoma multiforme (GBM). The immunosuppression observed in GBM is mediated by various mechanisms including the accumulation of myeloid-derived suppressor cells (MDSC), T-regulatory cells and tumor-associated macrophages (TAMs), immuno-suppressive cytokine milieu, and the expression of program death-ligand 1 (PD-L1) by tumor cells and MDSCs (Koyama, et al. 2016). These immunosuppressive mechanisms have contributed to the modest efficacy of immunotherapies for GBM patients, suggesting that further research is needed to improve therapeutic efficacy (Garber, et al. 2016; Dine, et al. 2017).

Immune checkpoint inhibitors are at the forefront of immunotherapy development resulting in unparalleled success in cancer therapy due to their broad bioactivity across many tumor types (Curran, et al. 2010; McGranahan, et al. 2016). Among the immune checkpoint blocking strategies, the two most clinically successful to date are targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and the interaction between programmed cell death protein 1 (PD-1) and its ligand 1 (PD-L1). However, one of the hallmarks of GBM is tumor heterogeneity, which is mostly characterized by distinct genetic alterations that occur in individual tumors originating in the same organ, and

tumor cell plasticity as a new source of cancer stem cells, impeding the success of immune checkpoint inhibitors in the treatment of GBM (Wieser, et al. 2018).

In a disease such as glioma, tumor heterogeneity hinders immunotherapy through the activation of different immune checkpoints. This may be related to redundancy of activating and inhibitory molecules targeted by immune checkpoint inhibitors (Koyama, et al. 2016). Therefore, multiple inhibitors targeting more than one immune checkpoint pathway have been employed, significantly enhancing survival. Unfortunately, these therapies often cause severe immune-related adverse events, often leading to treatment discontinuation, hospitalization, or even death (Markman, 2018; Hoffmann and Del Mar 2015).Our research is focused on the development of immunotherapeutic vaccines against GBM (Olin, et al. 2014a; Ohlfest et al. 2013; Xiong, et al. 2016), and on methods to target the immunosuppressive CD200 protein. The CD200 immune checkpoint regulates the immune system through paired receptors inducing inhibitory and activation signals (Kojima, et al. 2016). The inhibitory signal is due to the specific binding of the native form of the CD200 protein to the inhibitory receptor, CD200R1 and CD200-like receptors termed activation receptors (CD200AR) (Wright, et al. 2003). However, in contrast to the inhibitory receptor, the

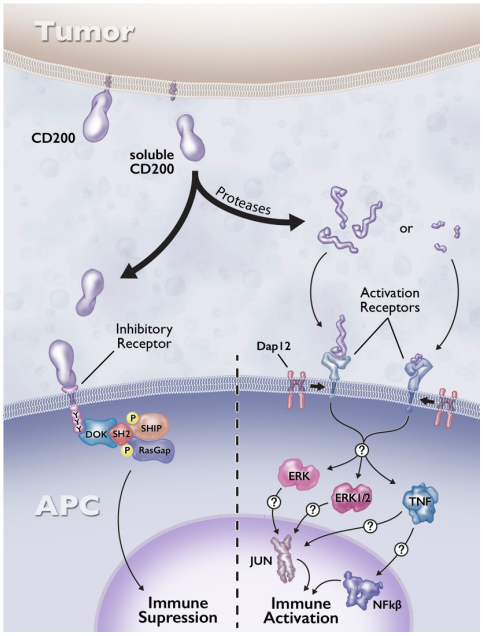


Figure 1. Experimental Model. CD200 is secreted from tumor cells, and binds to the CD200 inhibitory receptor, shutting down the immune system. However, CD200 is subjected to proteases, altering the folding and releasing peptide fragments exposing epitopes from the CD200 protein capable of binding to the CD200 activation receptors. The activation receptors have adjuvant-like properties and when stimulated, surmount the inhibitory signals activating the immune cells.

function and ligands inducing activation receptors have not been defined. There are four separate CD200AR genes in mice; CD200AR2, CD200AR3, CD200AR4, and CD200AR5, (Gorczynski, et al. 2004, Voehringer, et al. 2004), and two in humans; CD200RL1 and CD200RL2 (Wright et al. 2003). Although the CD200AR have not been well characterized, Gorczynski reported that specific peptide sequences bind to CD200ARs that contribute an activation signal attenuating the activation of leukocytes (Gorczynski, 2005; Gorczynski, et al. 2008) (Figure 1).

Several rigorous studies have provided evidence that targeting CD200 will enhance immunotherapy (Rygiel, et al. 2012; Copland, et al. 2007). We hypothesize that targeting the CD200 checkpoint will surmount the immune suppression produced by the tumor-derived CD200 protein. We developed peptide ligands to target the CD200AR (CD200AR-L). Peptides have advantages over proteins and antibodies as drug candidates because of their ability to penetrate further into tissue (McGregor, 2008), higher activity per unit mass, greater stability, and reduced potential for nonspecific binding resulting in decreased toxicity (Ladner, et al. 2004). Peptides are increasingly being developed to treat cancers including Cilengitide for GBM, Zaltrap for colon and Degarelix for prostate cancer. A recent search on *ClinicalTrials.gov* revealed over 50 actively recruiting or completed trials using peptide vaccines, 13 of which targeted CNS tumors supporting the feasibility of this approach.

We reported that the use of CD200AR-L in addition to tumor lysate vaccines significantly enhanced survival in a murine glioma model (Xiong, et al. 2016) and survival in a pilot trial in pet dogs with spontaneous high-grade glioma (Olin, et al. 2019). Specific binding of the CD200AR-L to the various receptor(s) remained unknown.

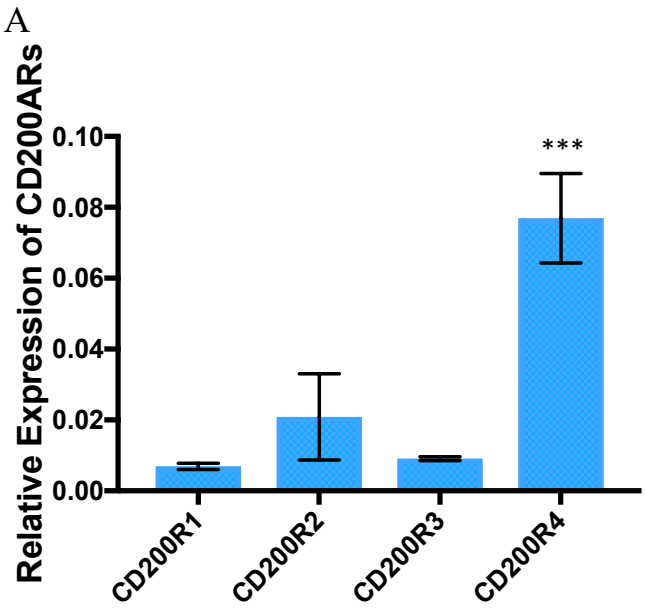
Therefore, we sought to use a macrophage cell line to knock out the CD200ARs. The binding of ligands to CD200ARs eliciting an immune response need to be determined. In this chapter, we report the development of a monocyte cell lines in which we systematically knocked out CD200ARs to generate macrophages expressing different CD200 receptors, singly or in combination. These cell lines allowed us to demonstrate binding between the CD200AR-L and specific CD200ARs and validate cell activation.

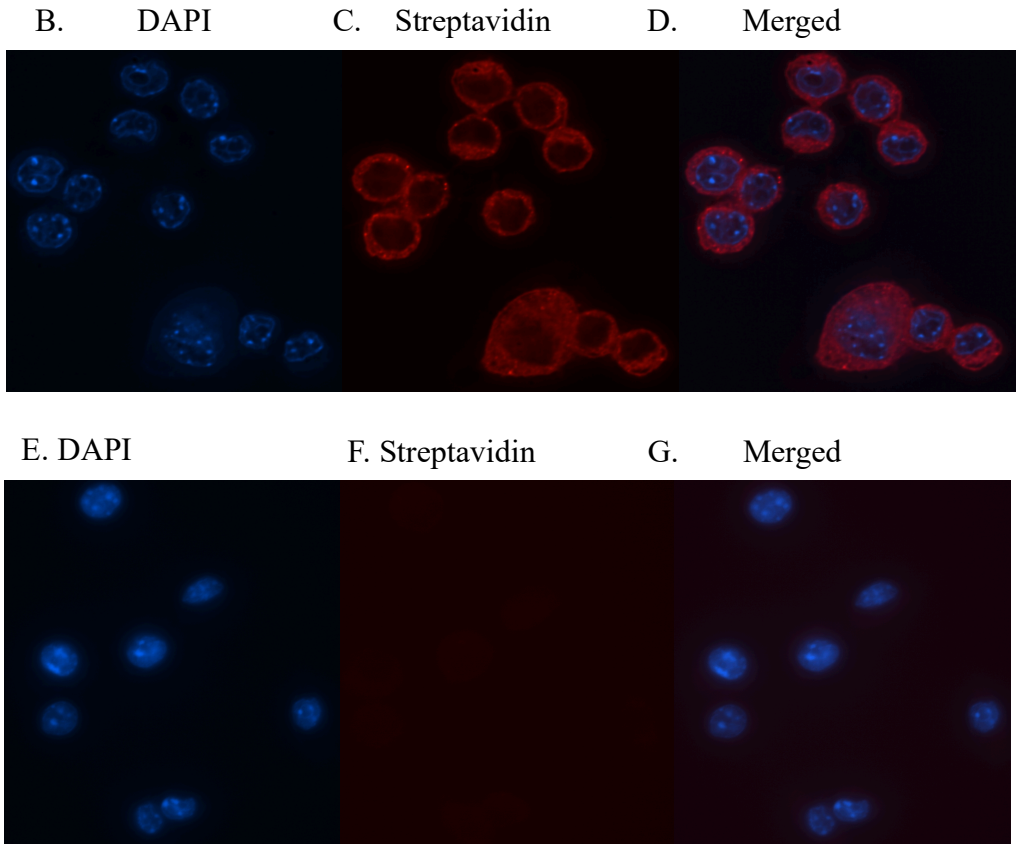
3.2 Results

3.2.1 Characterizing the Macrophages (Raw 264.7 M∅s)

To initiate our studies, we first characterized a macrophage (Raw 264.7) cell line. In these studies, RNA was isolated from M∅s to analyze the constitutive levels of the different CD200 receptors (Figure 2A). We confirmed that Raw 264.7 M∅s express all of the CD200 receptors. Next, we wanted to demonstrate that the peptide would bind to the cells. To accomplish this, we developed a biotinylated CD200AR-L (B-CD200AR-L) peptide to pulse macrophages and following incubation, cells were stained with a streptavidin alexafluor-568 conjugate and analyzed with microscopy (Figures 2B-D). Un-pulsed cells stained with Alexafluor-568 conjugate were used as a control (Figures 2E-G). We saw fluorescence on the cell surface of the M∅s pulsed with B-CD200AR-L, but no fluorescence was noted on stained M∅s with pulsed with non-biotinylated CD200AR-L or the un-pulsed controls. These findings validate that CD200AR is located on the M∅ cell surface and binds CD200AR-L. To test if biotinylation affected activity, wildtype macrophage cells were pulsed with the B-CD200AR-L and the functional response was assessed. Macrophages pulsed with nonbiotinylated CD200AR-L and un-pulsed cells

were used as controls (Figure 2H). The cells pulsed with B-CD200AR-L induced TNFα production that was not significantly different than the cells pulsed with non-biotinylated CD200AR-L. This demonstrated the ability to use the B-CD200AR-L to identify the CD200ARs is associated with natural CD200AR-L. In conclusion, these findings demonstrated that the CD200ARs are expressed constitutively in M∅s, and that the B-CD200AR-L can be used to assess the peptide binding on the macrophage cell lines.





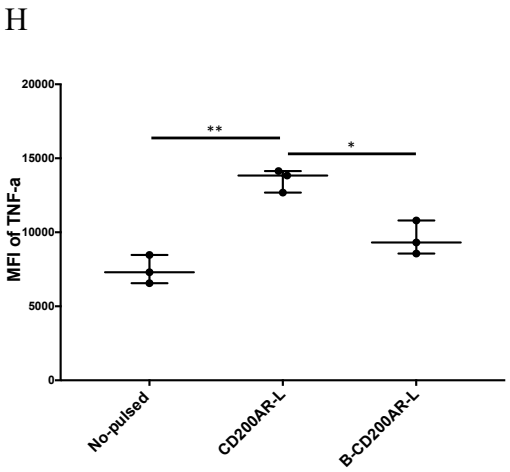
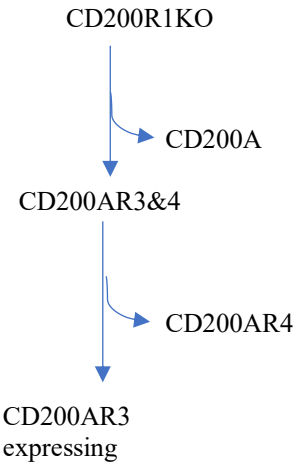


Figure 2. Characterizing the Macrophages (Raw 264.7 MØs). A) To determine the gene expression of CD200ARs, RNA from 10^6 unstimulated macrophages was analyzed using qPCR. The relative expression of each CD200ARs was calculated after being normalized with the housekeeping gene GAPDH. (B-G) Macrophage were pulsed with 10 uM biotinylated P1 (B-CD200AR-L) peptide, then cells were stained with streptavidin Alexa fluor 568. Stained MØs with streptavidin or DAPI alone were used as control (H) In a separated experiment, macrophages were pulsed with 10uM ligand

for 48h. Then, the supernatant was collected to determine TNF-a secretion. Error bars are ± SEM, asterisks represent a statistical significance *p < 0.05, **p = 0.005 or ***p = 0.0005 determined by multiple t test one per row.

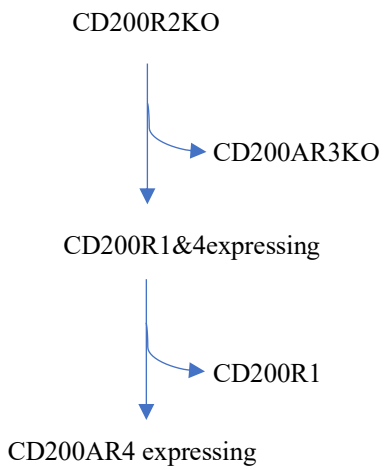
3.2.2 Generation of Knockout Macrophages

Now that we had a macrophage cell line to assess binding, CD200AR knockout cell lines were developed. To accomplish this, CRISPR guide RNAs (sgRNAs) were designed using online tools (http://genome-engineering.org) to target the exon 3 of either CD200R1 or CD200AR2, exon 2 of CD200AR3, and exon 4 of CD200AR4. Cells were transfected and sorted three times and sorted on a FACSAria II cell sorter (BD Biosciences, University of Minnesota Flow Cytometry Facility). PCR was used to confirm gene removal (Figures 3 C-E) and non-transfected cells were used as controls. First, a single CD200 receptors knockout were derived from the wildtype macrophages to get CD200R1, CD200AR2, CD200AR3, and CD200AR4 knock out, and from these, different macrophage cells lines described below were developed:

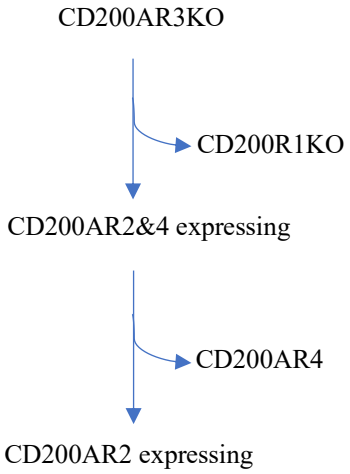


- SingleCD200R1KO (PCR validation Fig. 3A lane 1) cells were used to remove the CD200AR2 gene (PCR validation Fig. 3B lane 1) to derive CD200R1 CD200AR2 double KO cell line (expressing CD200AR3 and AR4). These cells were further used to knock out CD200AR4 (PCR validation Fig. 3C lane 5) resulting in a cell line that expressed CD200AR3 alone.

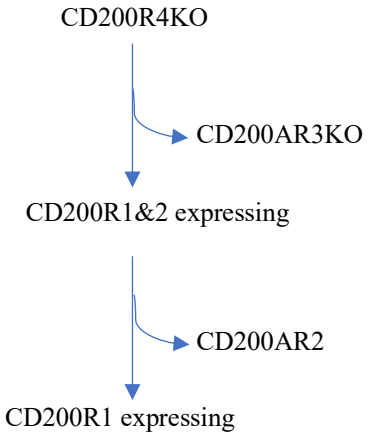
- Single CD200AR2KO (PCR validation Fig. 3A lane 3) cells were used to remove the CD200AR3 gene (PCR validation Fig. 3B lane 3) for the development of CD200AR2 and CD200AR3 double KO cell line (expressing CD200R1 and CD200AR). These cells were further used to knock out CD200R1 (PCR validation Fig. 3C lane 2) resulting in a cell line that expressed CD200AR4 alone.



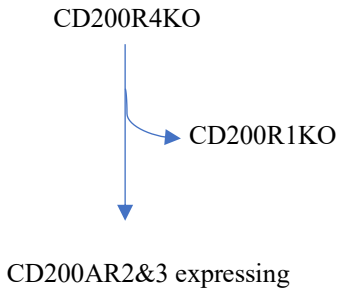
- Single CD200AR3KO (PCR validation Fig. 3A lane 4) cells were used to knock out the CD200R1 gene (PCR validation Fig. 3B lane 2) resulting in the development of a CD200R1 and CD200AR3 double KO cell line (expressing CD200AR2 and CD200AR4) that was then used to knock out the CD200AR4 gene (PCR validation Fig. 3C lane 5) creating a cell line expressing CD200AR2 alone.



- Single CD200AR4KO (PCR validation Fig. 3A lane 5) cells were used to knock out the CD200AR3 gene (PCR validation Fig. 3B lane 3) resulting in a CD200AR3 CD200AR4 double KO cell line (expressing CD200R1 and CD200AR2). This cell line was used to knockout the CD200AR2 (PCR validation Fig. 3C lane 3) to develop a cell line expressing CD200R1 alone.

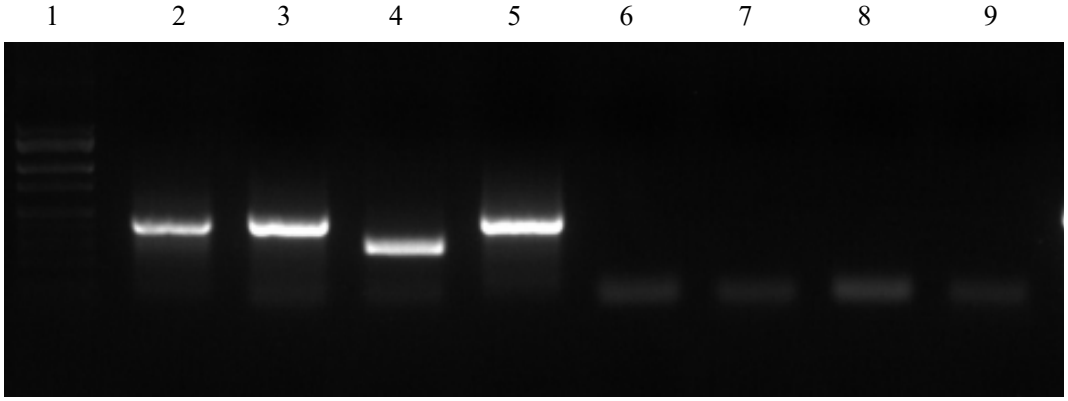


- Single CD200AR4KO (PCR validation Fig. 3A lane 5) cells were used to knock out the CD200R1 gene (PCR validation Fig. 3B lane 2) resulting in a CD200R1 CD200AR4 double KO cell line that expressed CD200AR2 and CD200AR3 (Figure 3C).



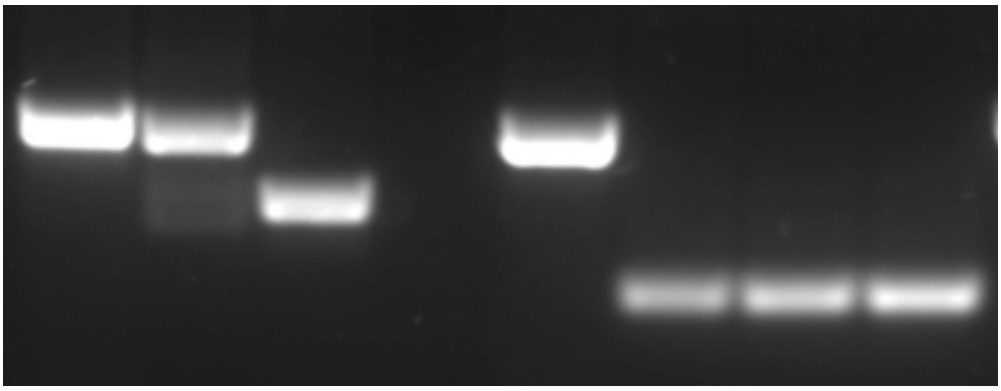
These experiments allowed us to derive the unique cell lines expressing different CD200 receptor. All receptor knockout cell lines were sequenced to validate gene removal. Additionally, all cells with receptors R1, AR3 or AR4 knocked out were validated by flow cytometry, however flow could not be done for the AR2KO since no antibody for CD200AR2 is available.

A

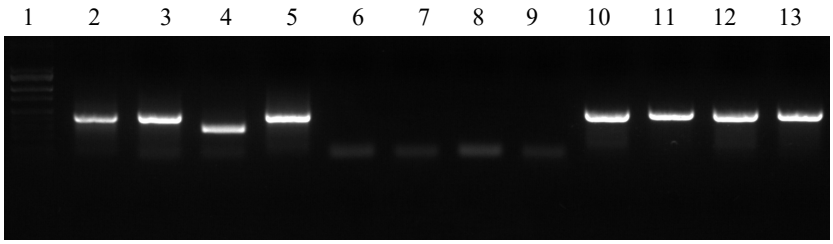


Lane	Lane
1 MW	
2. CD200R1	6. Control for CD200R1
3. CD200AR2	7. Control for CD200AR2
4. CD200AR3	8. Control for CD200AR3
5. CD200AR4	9. Control for CD200AR4

B 1 2 3 4 5 6 7 8



Lane	Lane
1 CD200AR2	5. CD200AR4.
2. CD200R1	6. CD200R1/AR2 GAPDH
3. CD200AR3	7. CD200R1/AR3 GAPDH
4. N/A	8. CD200R1/AR4 GAPDH



Lane	Lane
1 MW	7. Control for CD200AR2
2. CD200R1	8. Control for CD200AR3
3. CD200AR2	9. Control for CD200AR4
4. CD200AR3	10. CD200R1 GAPDH
5. CD200AR4	11. CD200AR2 GAPDH
6. Control for CD200R1	12. CD200AR3 GAPDH
	13. CD200AR4 GAPDH

Figure 3. Establishing the CD200 Receptor Knockout Macrophages. The system CRISPR/Cas9 was used to generate CD200 receptors knockout macrophages. A-C PCR validation was performed to determine the deletion of CD200AR genes.

3.2.3 Murine CD200AR-L Activates CD200AR2&3 and CD200AR3&4

With developing of a model to study the peptide CD200AR-L/CD200AR binding, we pulsed the macrophages with B-CD200AR-L. In contrast to the wildtype cells (Figure 4A), reduced peptide binding was seen on CD200AR2KO and CD200AR3KO and no peptide binding was observed on CD200AR4KO cells. Cells expressing different combinations of two CD200ARs. Were pulsed demonstrating strong peptide binding on these cells expressing CD200AR2&AR3, CD200AR3&AR4 and CD200AR2&AR4 (Figures. 4E&G).

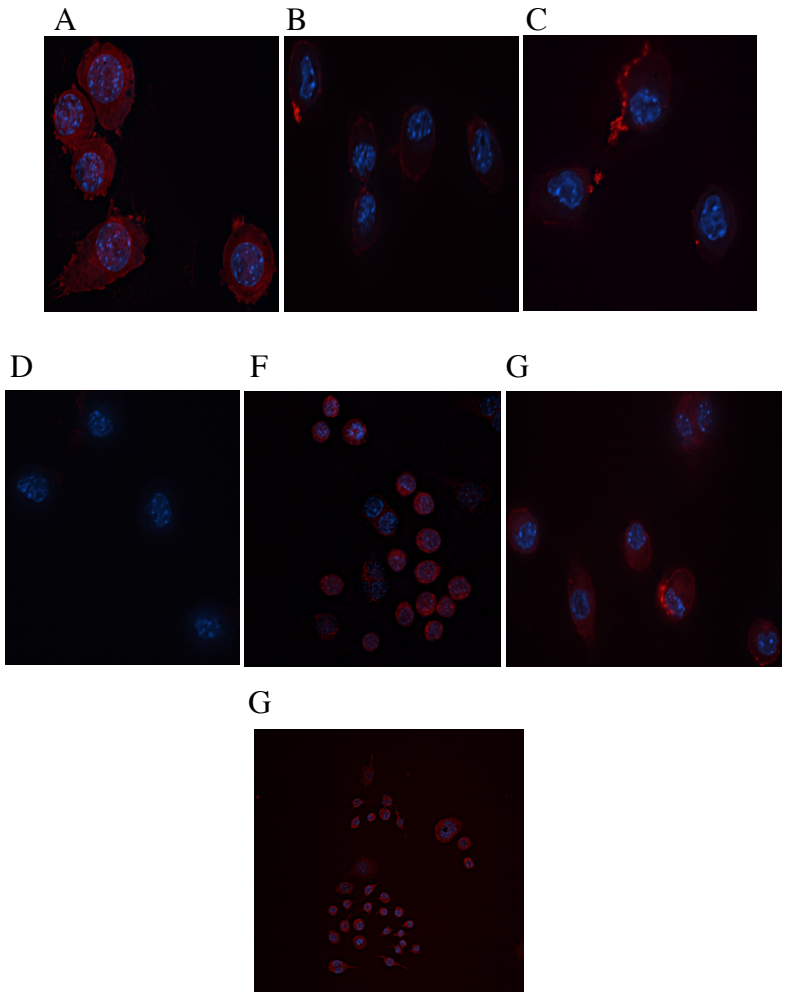
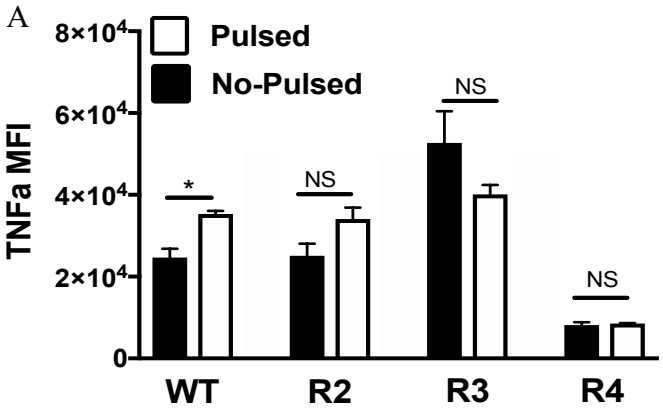


Figure 4. CD200AR-L Binds to CD200AR Complexes. (a) wildtype, (b) CD200AR2KO, (c) CD200AR3KO, (d) CD200AE4KO, (e) CD200AR2&3 expressing, (f) CD200AR-2&3 and (G) CD200AR2&4 expressing macrophage were pulsed with B-CD200AR-L and analyzed for binding.

3.2.4 CD200AR-L Primarily Signals through the CD200AR2&3 Complex

The functional effects of CD200AR-L binding to the different CD200ARs were determined by pulsing with CD200AR-L and analyzing supernatants for cytokine production. These experiments correlated with the binding experiments in that only the pulsed cells expressing a combination of two CD200 activating receptors induce TNFα secretion (Figure 5A), surprisingly, cells expressing the CD200AR2, 3 & 4 receptors do not induce TNFα secretion (data not shown). However, the CD200AR2&3 cells had a significant increase in IL-12 production ($p=0.012$) and both CD200ARs 2&3 and CD200AR2&4 cell lines had a significant increase in TNFα ($p=0.039$, $p=0.032$, respectively) and IL-6 ($p=0.006$, $p=0.02$, respectively) expression. In addition, only cells expressing CD200AR2&3 complexes had a significant increase of MCP-1 ($p=0.005$) (Figure 5B-E). The findings are shown here demonstrated that the ligand CD200AR-L primarily targets the CD200AR2&3 complex. However, there was also a lesser response by the CD200AR2&AR4 complexes activating antigen-presenting cells. It is known that activating immune receptors comprised of multiples subunits that can mix and match those subunits and allow a cell to construct varying receptor complexes with different ligand specificity. These results show that innate cells, like macrophages, build distinct CD200AR complexes with different binding affinities to maintain homeostasis of the immune system. This is an important mechanism of how the immune system balances immune responses through receptor diversity.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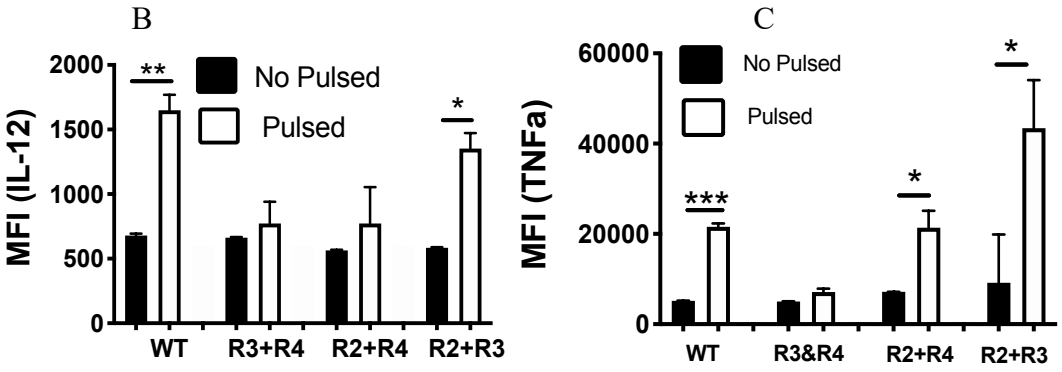
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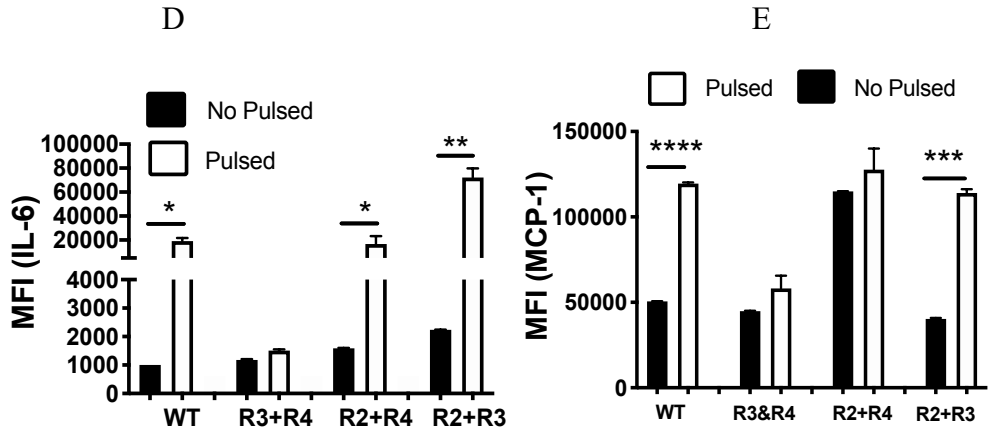
R2+R3


D
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MFI (MCP-1)
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Pulsed
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R3&R4
R2+R4
R2+R3

Figure 5. CD200AR-L Primarily Signals through the CD200AR2&3 Complex. (A) CD200AR macrophage cell line expressing single CD200ARs were pulsed with the CD200AR-L and analyzed for TNFα CD200 macrophage cell lines expressing duel CD200ARs were pulsed with the CD200AR-L, supernatants were analyzed for (B) IL-12, (C) TNFα, (D) IL-6 and (E) MCP-1. Error bars are representative of standard deviation (n=3/group $*P < 0.05$ and $**P<0.005$; by t-test.

3.3 Discussion

There have been contradictory reports regarding binding of CD200 to the CD200ARs and whether that the binding triggers immune activation. (Gorczynski, et al. 2004; Hatherley et al. 2005). Wright et al. (2003) reported that mCD200RLa (CD200AR4) and mCD200RLb (CD200AR3) do not bind mCD200. Additionally, Hatherley et al. (2005) showed proteins expressed at the cell surface and directly at the protein level that the soluble CD200 did not bind to CD200ARs. These two groups concluded that the ligands for CD200 activation receptors were unknown. Conversely, Gorczynski, et al. 2004 reported that soluble CD200 binds to CD200AR expressed in COS7 cells providing the first evidence that members of the CD200R family other than CD200R1 can bind CD200. Moreover, CD200-derived peptides may discriminate between CD200R1 or other CD200ARs and regulate inhibitory and activating functions, as defined by the immunological context (Gorczynski, et al. 2008). Overall, a better understanding of the ligation of CD200ARs expressed on myeloid cells and its immunological consequences is needed.

We have demonstrated receptor specificity of the CD200AR-L and the functional interplay between CD200AR complexes. These studies show that cells expressing CD200ARs 2&3 primarily responded to stimulation by CD200AR-L while cells expressing receptor combinations of 1,2&3, 1,3&4 or 2,3&4 failed to respond. In earlier

report, we described the development of three murine CD200AR-Ls based on previously published peptides (Xiong et al. 2016; Moertel et al. 2014) that provided different survival results in a murine glioma model. This may explain our observation that CD200-derived peptides bind with different ligand specificity to a duplex-complexes of CD200ARs and induce differences in a survival benefit in our breast carcinoma and glioma murine models. (Moertel, et al. 2014). This data shows that CD200ARs form complexes to interact with the peptide ligands to optimize the biological function of macrophages. It also shows that CD200-derived peptides function protectively in our mouse model of glioma because the CD200AR-L binding to a complex of two CD200 activation receptors on innate immune cells like macrophages so induces immune activation that could overcome the negative signal of the CD 200 protein. This data is in agreement with previous reports that CD200-derived peptides designed to target the different CD200ARs modulate inflammatory processes *in vivo* in a selective manner (Gorczynski, et al. 2008).

3.4 Materials and Methods

Transfection: Raw 264.7 M∅s were transfected using Neon electroporation system (Invitrogen). Briefly, Raw 264.7 M∅s were grown in RPMI 1640 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and incubated at 37^0C until 80% confluent. Cells were harvested by trypsinization, washed once in RPMI and resuspended in 1X PBS at a concentration of $5e10^6$ cell density (cells/ml). Then, 10ul cells were taken for the transfection. Ten ul of suspension cells was precipitated and resuspended in 10 ul Neon Buffer R, mixed with 1 ul (1ug/ul) Clean-Cap Cas9 mRNA (TriLink

Biotechnologies) and 1 ul (100 pmol/ul) CRISPRevolution sgRNA Synthego (Synthego) and incubated for 2 min. Transfection conditions were 1,720 pulse voltage, 10 pulse width, and 2 pulse numbers. Two days after transfection, a PCR was performed, and the PCR products were sequenced to validate the deletion. Cells were assessed for expression status by flow cytometry. CD200R1 was assessed by staining with PE-conjugated OX-110 (Biolegend), and CD200AR3 was assessed by staining with APC-conjugated Ba13 (Biolegend).

Immunofluorescence Cell-Binding assay: 5 $X10^4$ macrophage were grown in a Lab-Tek II 8 chamber slide in 200 ml RPMI containing 10% calf serum and 1% penicillin/streptomycin. Following ~70% confluency, cells were washed twice with 1X PBS and pulsed with 10uM biotinylated peptide ligand for an hour fixed in 4% PFA for 20 min at RT then incubated with streptavidin alexafluor 568 conjugate (Thermo Fisher Scientific) for 1 h, washed with 1X PBS and stained with 1ug DAPI. Imaging was performed using the Nikon Inverted TiE Deconvolution Microscope System (University of Minnesota).

Cytokine Secretion: Unless otherwise stated, all cells were treated at the same conditions for the cytokine secretion. A total of 1 × 106 cells was grown in 0. 500 ml in RPMI-1640 in a 48-well plate for 12 h, then pulsed with 10uM ligand P1, and incubated for an additional 48 h. LPS (1uM) and non-pulsed cells were used as controls. Supernatant (50ul) were collected and analyzed for TNFα levels using cytometry bead array (Biosciences). Data was analyzed using Flowjo v10.

Chapter 4

Unraveling the Signaling Pathways of the CD200AR

4.1 Introduction

Paired receptors are cell surface proteins expressed primarily on immune cells containing conserved extracellular domains capable of eliciting either inhibitory or stimulatory signals (Humphrey et al. 2005). The inhibitory receptors have long immunoreceptor tyrosine-based inhibitory motifs (ITIM) within the cytoplasmic region (Daeron, et al. 2008). Conversely, activation receptors usually have short cytoplasmic tails. These short tails contain positively charged lysine or arginine residues in their transmembrane domain to associate with adaptor proteins possessing immunoreceptor tyrosine-based activation motifs (ITAM) including DAP12, CD3, CD79, and FcR that provide tyrosine-based activation motifs as docking sites for downstream signaling (Isakov, et al. 1997). In addition to the ITAM signal, there is a tyrosine-based signaling motif (YINM), which is present in the adaptor protein DNAX-activating protein of 10 kD (DAP10) and it is known that DAP10 coop with some immune activation receptors like NKG2D, Ly49H, and Ly49D.

Unlike the inhibitory receptor, CD200R1, CD200ARs have a positively charged lysine residue within their transmembrane-spanning regions, may recruit signaling molecules directly, and are thought to function by coopting accessory molecules (Wright GJ, et al. 2000, Wright, et al. 2003, Kojima, et al. 2007). *In vitro* studies have shown that two CD200 activation receptors, CD200AR4 and CD200AR3, coopt with the adaptor protein, DAP12, suggesting that these receptors could transmit strong activating signals (Wright et al. 2003; Voehringer, et al. 2004). However, a connection between recruitment

of the CD200ARs and DAP12 has not been established (Voehringer, et al. 2004). Therefore, the role of DAP12 or other adaptor protein relative to CD200 activating immune receptor needs further investigation.

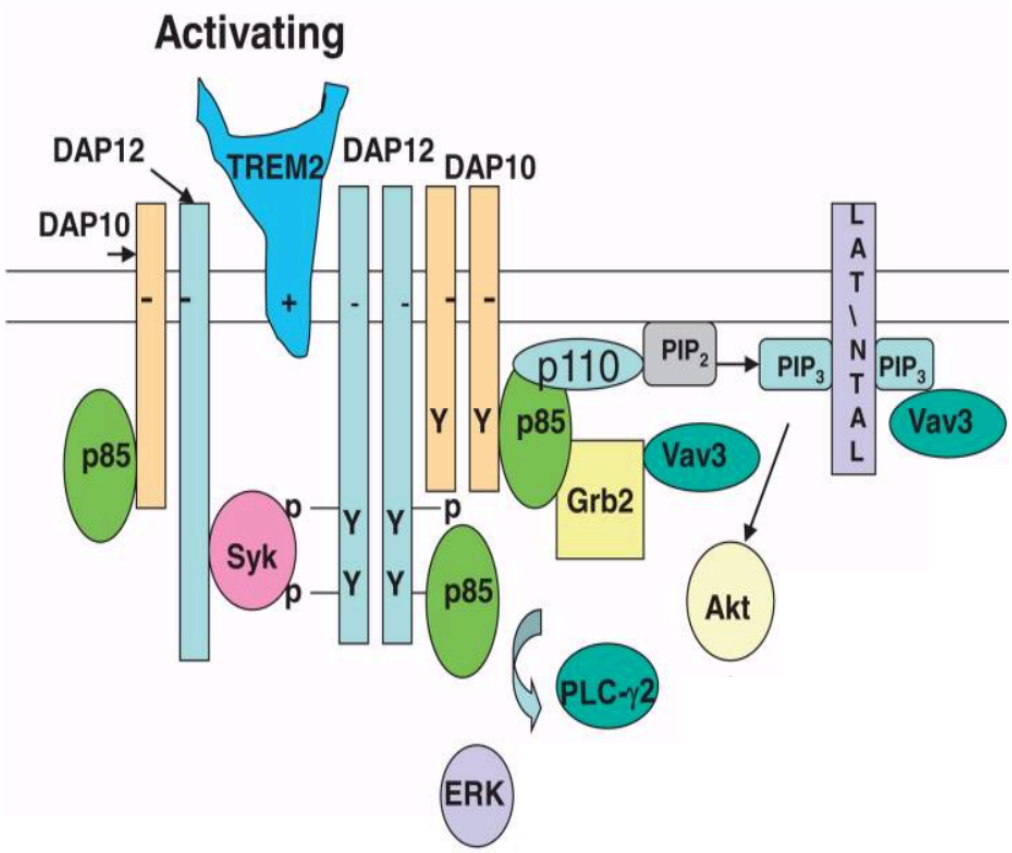


Figure 1. Proposed DAP12 Signaling Pathway in Macrophages and Osteoclasts. In response to ligation of TREM2, Src family kinases phosphorylate the ITAM of DAP12 and the YINM motif of DAP10, which form docking sites for the Syk and p85, with the subsequent recruitment of PLC-γ2 and Grb2. This signaling complex then leads to the activation of Akt, ERK1/2, and Vav3. Simultaneously, SHIP1 is recruited to the DAP12 ITAM where it may dislodge or prevent the further recruitment of SH2 or SH3 domain–containing proteins, including p85 and Syk respectively. This leads to an arrest in the activation of PI3K, ERK, Akt, and VAV3. Peng Q, et al. 2010.

The initial studies into the signaling pathways involved with the CD200AR-L/CD200AR binding focused on DAP10 due to its association with the DAP12 pathway (Lanier et al. 2009). DAP10 is a transmembrane signaling adaptor that has a short extra-cytoplasmic domain and no known ligand-binding properties and is predominantly expressed in immune cells, including NK cells, T cells, and monocytes (Figure 1) (Wu et al. 1999; Chang et al. 1999). DAP10 does not have a cytoplasmic ITAM, but it displays an YINM motif that couples to phosphatidyl-inositol 3-kinase (PI3K) dependent pathways (Nicolas et al. (2003). Moreover, it is reported that human NKG2D-DAP10 triggers cytotoxicity (Wu et al. 2000; Billadeau et al. 2003) and mediates primary stimulation signals in addition to co-stimulatory signals in NK cells (Billadeau et al. 2003; Zompi et al. 2003).

DAP12 is a 12-kD homodimer plasma membrane that associates with numerous receptors through paired charged amino acid residues within the transmembrane domains (Peng et al. 2010). In myeloid cells, several DAP12-associated receptors have been identified. These receptors fall into two categories: members of the immunoglobulin domain superfamily, such as triggering Receptor Expressed on Myeloid cells 1 (TREM-1), triggering Receptor Expressed on Myeloid cells 2 (TREM-2), triggering Receptor Expressed on Myeloid cells 3 (TREM-3), myeloid-associated immunoglobulin-like receptor II (MAIR-II), CD200 activation receptor 4 (CD200RLa), signal-regulatory protein beta (SIRP-β) and paired immunoglobin-like type 2 PILR- β; and members of the C-type lectin family, such as myeloid DAP12-associating lectin 1 (MDL-1) and mouse NKG2D-short (Lanier et al. 2019, Peng et al. 2010).

Some studies showed that DAP12 is required to down-regulate Toll-like receptor (TLR)–induced production of cytokines to limit inflammatory responses *in vivo* (Hamerman et al. 2005, 2006) suggesting that DAP12 may have both inhibitory and activating functions. Previous reports have shown that CD200ARs pair with DAP12 but they failed to demonstrate an effector immunological function. Thus, whether DAP12 inhibits or activates the immune biological functions of the CD200Ars is unknown.

Intracellularly, DAP12 does not have signal-transducing elements other than a single ITAM, which recruits and activates Syk in myeloid cells following tyrosine phosphorylation and Syk and ZAP70 in NK cells (Lanier et al. 2008). Moreover, like the CD3 and CD79 subunits, DAP12 has an acidic amino acid (aspartic acid) embedded within its transmembrane region allowing stable, non-covalent complexes with its associated receptors (Lanier et al. 2009).

In summary, in the previous chapters, we demonstrated that a peptide ligand (CD200AR-L) bind to CD200AR complexes leading to the activation of macrophages and cytokines secretion, however, the signaling pathways that result in immune activation remain unknown. Therefore, we performed ingenuity pathway analysis (IPA) on wildtype naïve-CD11b cells pulsed with CD200AR-L. The results of this analysis, in conjunction with current literature, led to the development of the hypothesis that CD200 activation receptors recruit and signal through the DAP10 and DAP12 pathways. In this chapter, we will test our hypothesis by addressing these specific aims: i) interrogate at transcriptional level the response of DAP10 and DAP12 over time in wildtype CD11b cells pulsed with CD200AR-L, ii) assess for the presence of the downstream signaling molecules of DAP10 pathway and their ability to knock down transcription using selected inhibitors,

iii) we suggest that the CD200AR may pair with DAP10 or DAP12 or both to activate antigen-presenting cells since DAP10 and DAP12 have been reported to form complexes following activation, hence, we will assess whether complexing of DAP10 and DAP12 occurs after CD200AR ligation, and iv) assess the *in vitro* and *in vivo* the effects of DAP10 and DAP12 pathways.

4.2 Results

4.2.1 Ingenuity pathway Analysis (IPA) Suggests CD200AR-L Signals through the DAP10 Pathway

Following optimization of the CD200AR-L (described in chapter 2), we wanted to determine the signaling pathway utilized by the CD200ARs. To accomplish this, CD11b cells isolated form wildtype mice were pulsed with the CD200AR-L for 1h. Data was analyzed using (IPA) (Qiagen) and revealed an upregulation of multiple signaling molecules downstream of DAP10 leading to the development of our experimental model shown in Figure 2. These experiments suggest that the ligand CD200AR-L induces of activation DAP10 signaling pathway on monocytes/antigen-presenting cells.

A

Molecule	The CD200-derived peptide
Dock8	Upregulated
ERK 1/2	Upregulated
TNF	Upregulated
IL-6R	Upregulated
IRAK	Upregulated
IRAK (phosphoralated)	Upregulated
JAK 1	Upregulated
JAK 2	Upregulated
C-jun	Upregulated
p38 MAPK	Upregulated
TIRAP	Upregulated
TRAF2	Upregulated
IKK complex	Upregulated
VAV1	Upregulated
RIPK2	Upregulated
TRAPP	Upregulated
ADAM 15	Upregulated
PRKCD	Upregulated
NOD2	Upregulated
NOD1	Upregulated
PYCARD	Upregulated
NFk-kB1-RelA	Upregulated
NFkB (complex)	Upregulated
PI3K (complex)	Upregulated

B

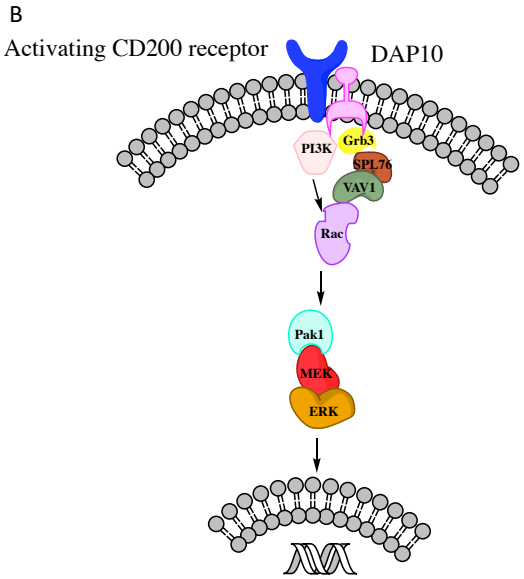


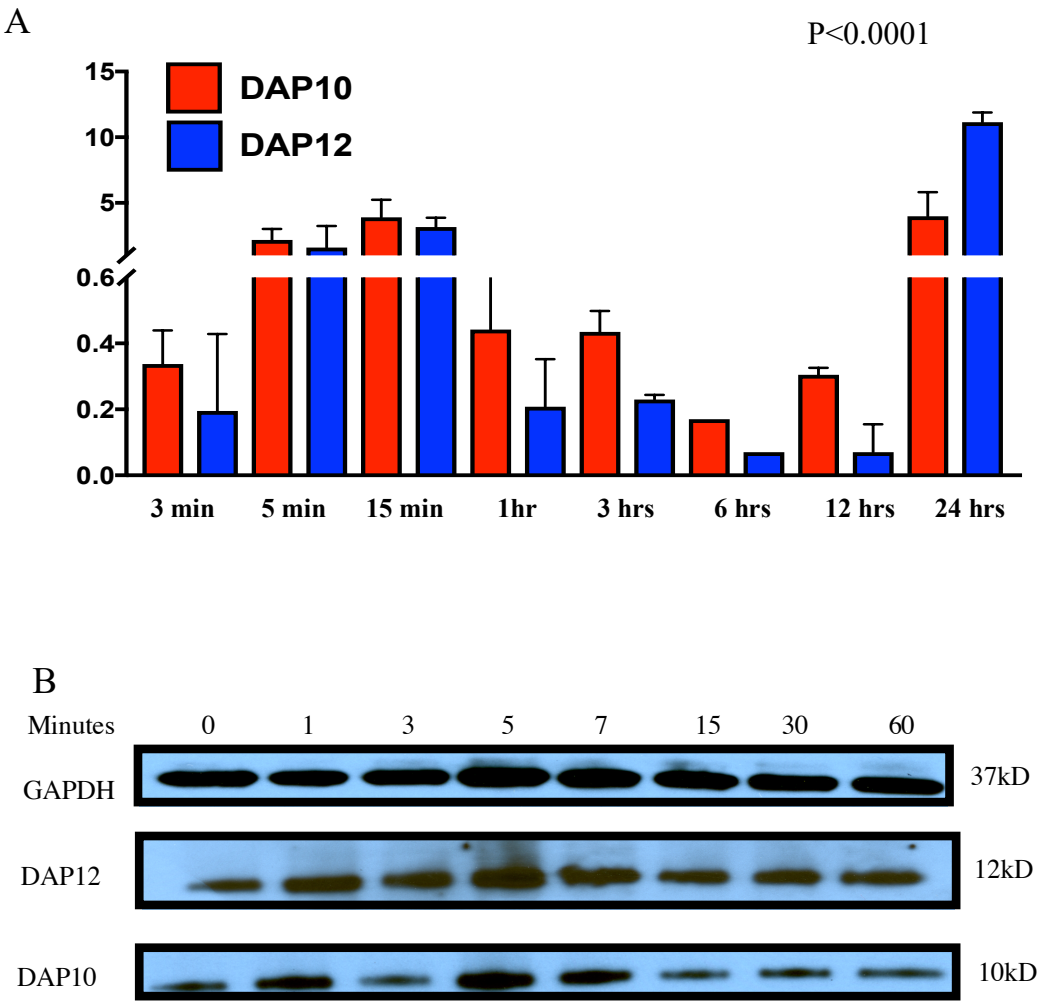
Figure 2. The CD200 Derived Ligand CD200AR-L activates the Canonical DAP10 Signaling. CD11b cells isolated from wildtype mice, pulsed with 10uM CD200AR-L for one hour. Then RNA was harvested and analyzed using NanoString technology. Data analysis and interpretation to identify the immune pathways were done using ingenuity pathway analysis (IPA) (table 1), and immune molecules DAP10 related are highlighted on a red-square (A) and leading to our experimental model of the immune activation of APC/macrophages (B).

4.2.2 CD200ARs Signal through Dap10 and DAP12 Molecules.

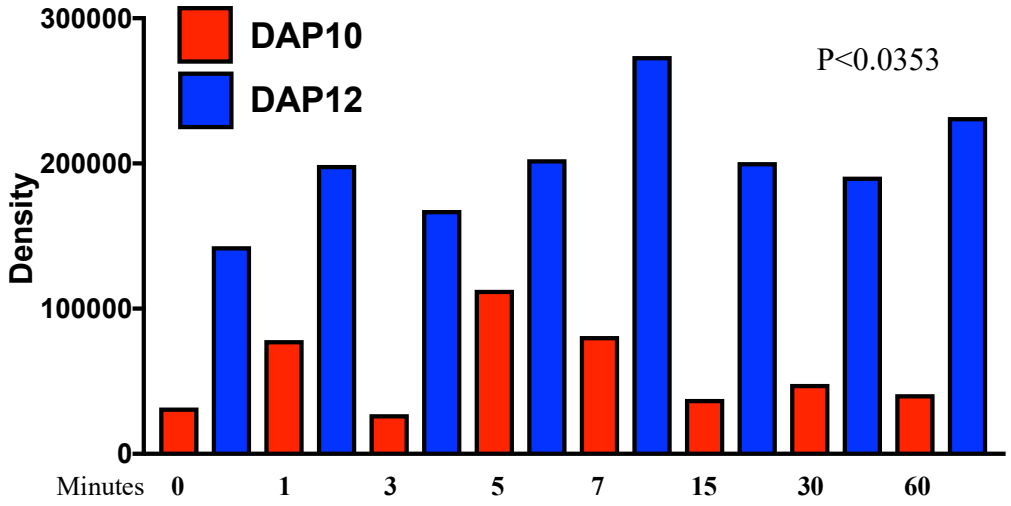
We next wanted to validate the IPA analysis suggesting that CD200AR stimulation could initiate DAP10 or DAP12 signaling pathways. According with the gene expression profile results, CD200AR-L induced gene expression of DAP10 and DAP12 for 1 h then cells send re-stimulation signals upregulating DAP10 and DAP12 12 hours later (Figure 3A). To validate transcription expression, in separate experiments, CD11b cells isolated from wildtype mice were pulsed with the CD200AR-L at 1, 3, 5, 7, 15, 30 and 60 mins and analyzed for protein levels of DAP10 and DAP12 (Figure 3B). To

quantitate these results, density was measured (Figure 3C) and normalized compared to non-pulsed (Figure 3D). Results were consistent with the model where, immune-activating

receptors recruit DAP10 and DAP12 molecules to deliver positive signals since DAP10 and DAP12 have a short cytoplasmatic tails with no activation motif.



C


DAP10
DAP12
P<0.0353
Density
300000
200000
100000
0
Minutes 0 1 3 5 7 15 30 60

D

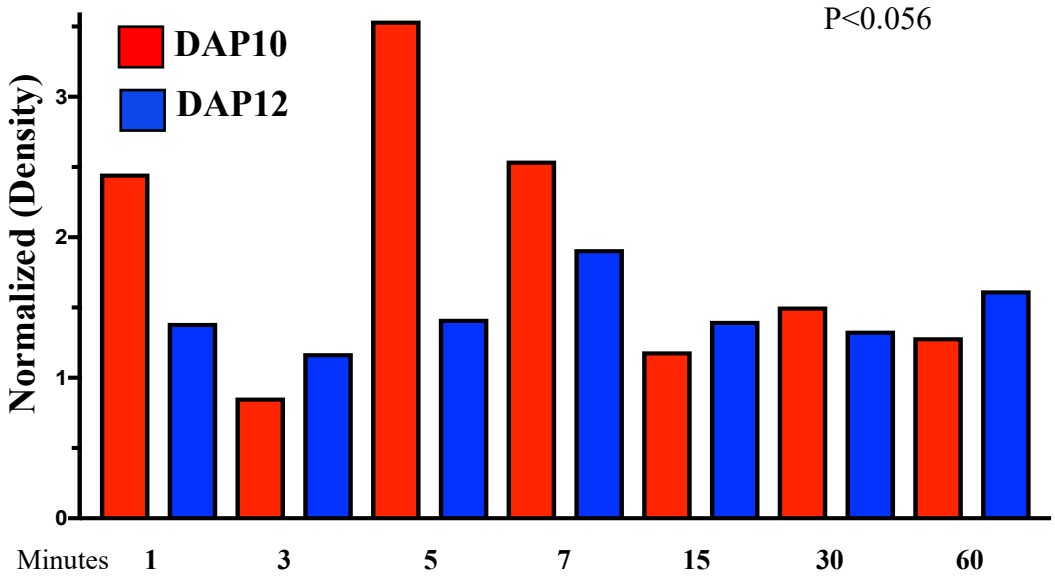

DAP10
DAP12
P<0.056
Normalized (Density)
3
2
1
0
Minutes 1 3 5 7 15 30 60

Figure 3. CD200AR-L Induces DAP10 and DAP12 Molecules. CD11b cells isolated from wildtype mice were pulsed with 10 uM CD200AR-L at different time frame and analyzed for (A) DAP10 and DAP12 translation levels and (B-D) validated by western analysis. P value determined by one-way ANOVA.

4.2.3 CD200AR-L Activates the DAP10 Cascade

To further validate our studies, we next evaluated the gene expression profile of the DAP10 signaling molecules upon stimulation of APC/macrophages with CD200AR-L1. To accomplish this, wildtype cells were pulsed for 6 hrs with the CD200AR-L, RNA was extracted and analyzed for expression of downstream signaling molecules (Figure 4A). We confirmed upregulation of the expression of DAP10 signaling molecules in murine CD11b, SLP76 and VAV1, which are functional effector downstream of DAP10, were mainly up-regulated. These experiments demonstrated the activation of signaling molecules downstream of the DAP10/DAP12 pathways. Next, we designed an inhibition assay to determine specific signaling molecules downstream of the DAP10/DAP12 pathway, CD11b cells were stimulated with 10uM CD200AR-L +/- theCD200AR-L (10uM) P38MAPK inhibitor (SB203580) (Figure 4 G-I) or (10nM) Jak1/Jak3 inhibitor (Tofacitinib) (Figures 4J-K), and gene expression analyzed at 0–6h and 0-18h by quantitative PCR. As expected from the signaling data reported above, gene expression of P38MAPK, ERK1/2, Vav1, SLP76, and Jak1/Jak3 was inhibited. These data demonstrated that CD200AR can trigger CD11b activation by a mechanism dependent on MAPK kinases. Taking together, these findings suggest that CD200ARs control CD11b cell activation via a previously unknown regulatory pathway that is, a PI3-K-dependent,

coupling of the receptor to the downstream effectors Vav1, Rho family GTPases and ERK1/2.

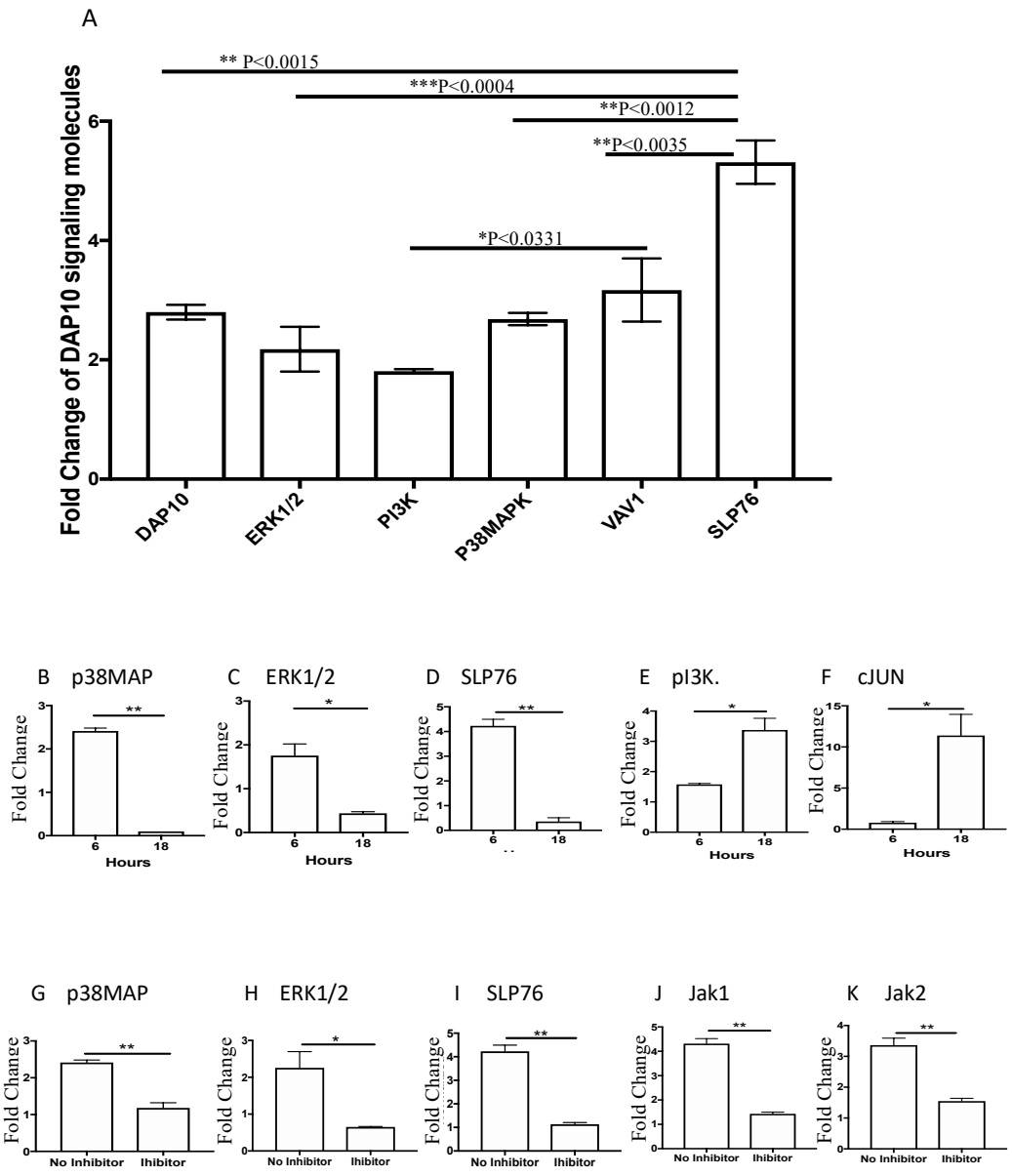
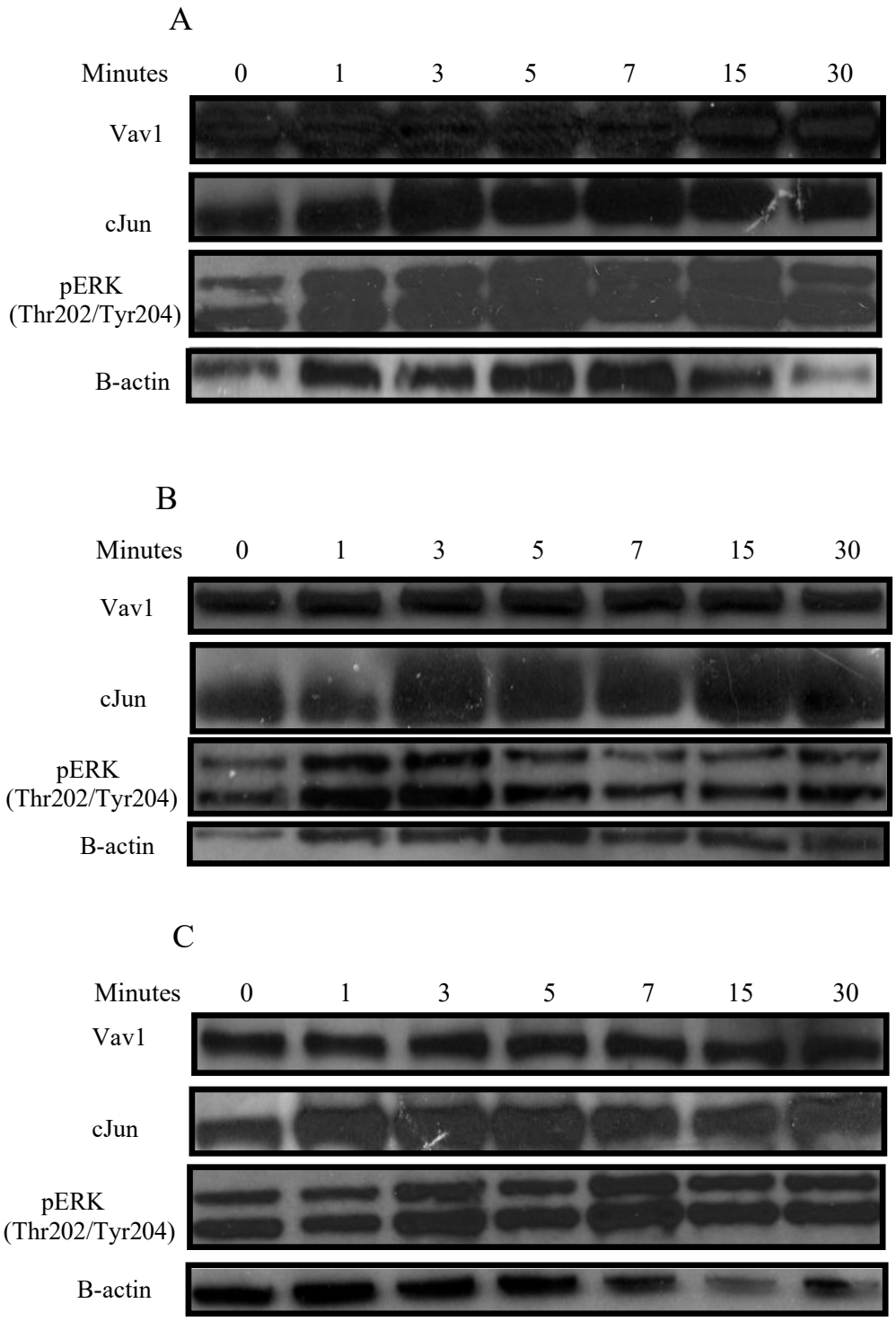


Figure 4. CD200AR-L Activates the DAP10 Cascade. CD11b cells isolated from wildtype mice were pulsed with P1CD200AR-L for 6h, (A) RNA was isolated and analyzed for various signaling molecules downstream of the DAP10 pathway. In separate experiments, CD11b cells pulsed for 6 or 18 hrs, transcription levels were analyzed for (B) p38MAP, (C) ERK1/2, (D) SLP76, (E)pI3K or (F)

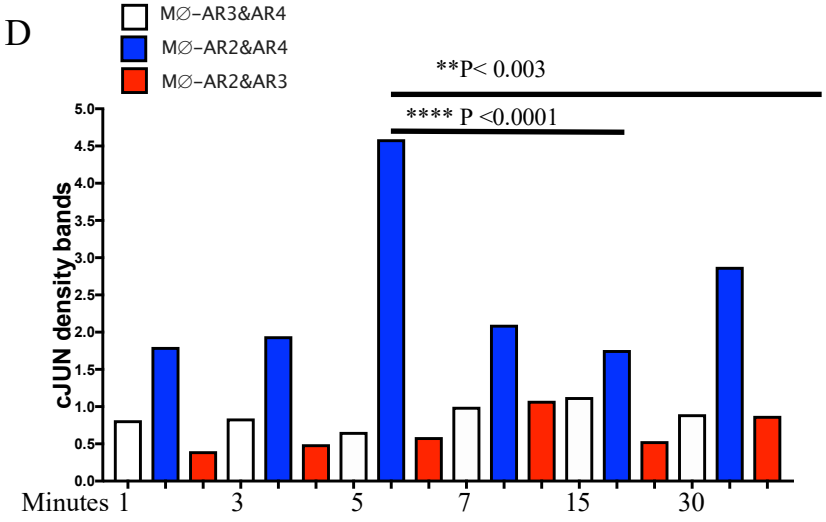
cJUN. In separate wells, cells pulsed for 6 hours with the P1CD200AR-L + the inhibitor SB203580 and analyzed for transcription levels of (G) p38MAP, (H) ERK1/2, (I) SLP76 or the CD200AR-L + Tofacitinib and analyzed for transcription levels of (J) Jak1 and (K) Jak2. Error bars are representative of standard deviation (n=3/group *P < 0.05 and **P<0.005; by t-test.

4.2.4 DAP10 and DAP12 Primary Signal through the CD200AR2&3 Complex

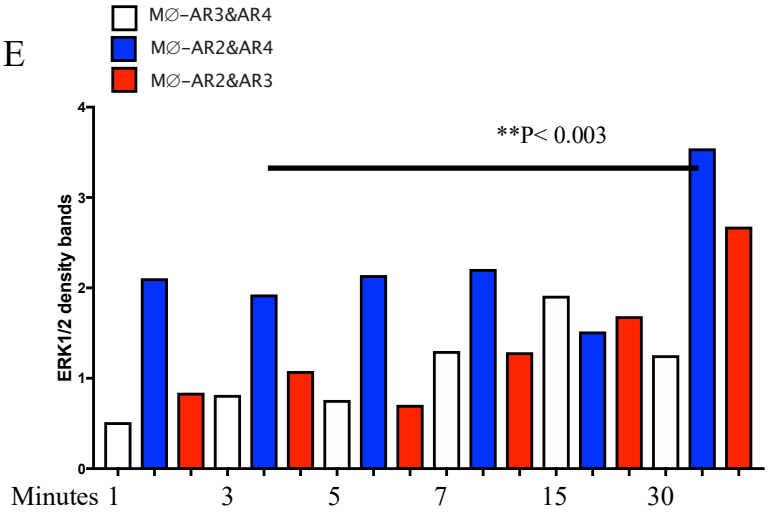
We next asked which CD200ARs primarily signal through the DAP10 or DAP12. To accomplish this, we took CD200AR cells expressing the 2&3, 2&4 and 3&4 complexes and pulsed them with the CD200AR-L. We observed by western analysis that ERK ½ signaling was primarily activated through the CD200AR2&3 complex (Figure 5A). Therefore, we repeated it looking at Vav1 and c-Jun (Figure 5B&C) since Vav1 has associated to DAP10 pathway showing the signaling was mainly through the CD200AR2&3 complex for Vav1, both CD200AR2&3 and CD200AR2&4 activated c-Jun. This may be due to a separate and nucleus signaling and needs further investigation.


A
Minutes 0 1 3 5 7 15 30
Vav1
cJun
pERK
(Thr202/Tyr204)
B-actin
B
Minutes 0 1 3 5 7 15 30
Vav1
cJun
pERK
(Thr202/Tyr204)
B-actin
C
Minutes 0 1 3 5 7 15 30
Vav1
cJun
pERK
(Thr202/Tyr204)
B-actin

D


MØ-AR3&AR4
MØ-AR2&AR4
MØ-AR2&AR3
**P< 0.003
**** P <0.0001
cJUN density bands
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Minutes 1
3
5
7
15
30

E


MØ-AR3&AR4
MØ-AR2&AR4
MØ-AR2&AR3
**P< 0.003
ERK1/2 density bands
4
3
2
1
0
Minutes 1
3
5
7
15
30

F

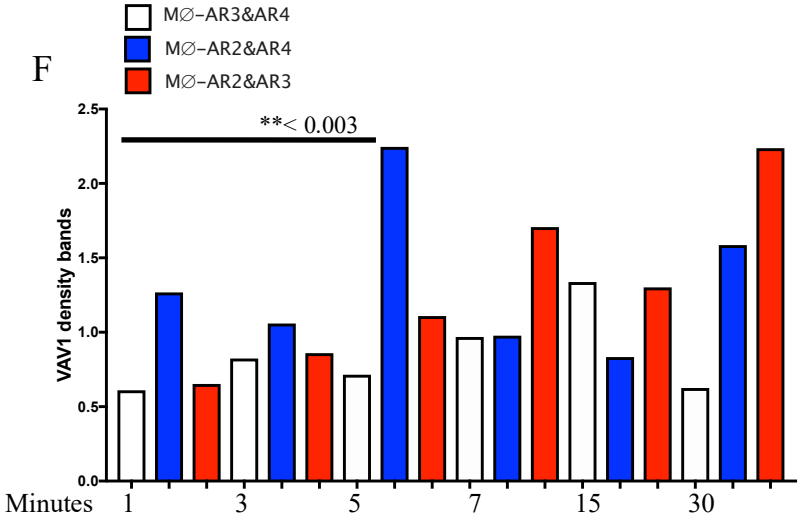
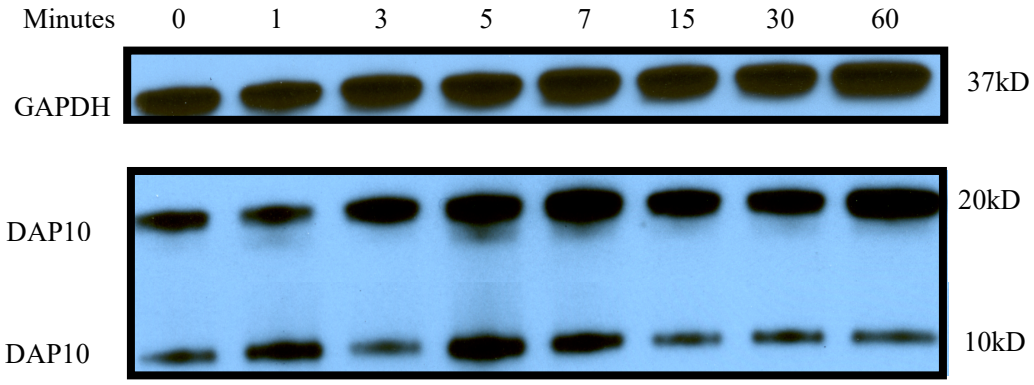

MØ-AR3&AR4
MØ-AR2&AR4
MØ-AR2&AR3
**< 0.003
VAV1 density bands
2.5
2.0
1.5
1.0
0.5
0.0
Minutes
1
3
5
7
15
30

Figure 5. DAP10 and DAP12 Primary Single through the CD200AR2&3 Complex. Macrophage expressing CDAR 2&3 (A), 2&4(B) and 3&4 (C) complexes were pulsed with the CD200AR-L. Cells were (A-C) analyzed by western analysis, and density bands corresponding to c-JUN (D), ERK1/2 (E), VAV1 (F) were measured. Error bars are representative of standard deviation (2=3/group *P < 0.05 and **P<0.005; by t-test).

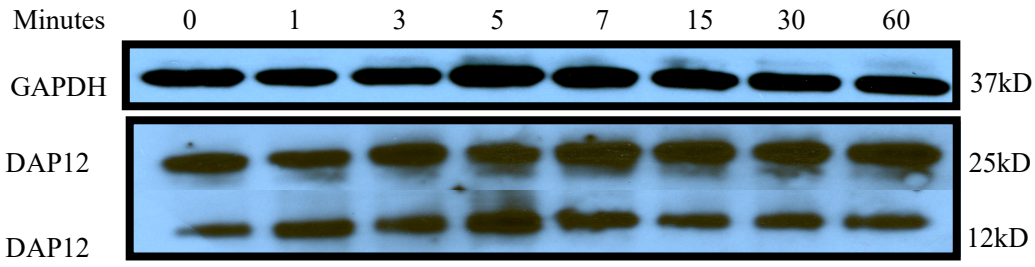
4.2.5 Dap10/Dap12 Complex together

It has reported that DAP10 forms complexes with DAP12 and coopt with immune-activating receptors to signal downstream and activate innate cells. Therefore, we assessed whether DAP10/DAP12 forms complexes after CD200AR-initiated signaling. In these experiments, CD11b cells from wildtype and DAP10 KO mice were pulsed with the CD200AR-L at different times and analyzed for DAP10 (Figure 6A) or DAP12 (Figure 6B) by western analysis. These experiments revealed a DAP10 and DAP12 band at both 20kD and 24kD respectively but was gone in the DAP10KO CD11b cells. To determine if this is a doublet or DAP10/DAP12 complex, CD11b cells isolated from DAP10KO mice were analyzed for DAP12 by western (Figure 6C). We observed tissue from DAP10KO mice failed to form a complex demonstrating a DAP10/12 complex. These findings are in agreement with previous reports, the DAP10/DAP12 complex coop with immune-activating receptors (Lanier LL, et al. 2009). Moreover, by measuring the densities of the western bands revealed that the removal of DAP10 inhibited the activation of DAP12 pathway (Figure 6D).

A


Minutes
0
1
3
5
7
15
30
60
GAPDH
37kD
DAP10
20kD
DAP10
10kD

B


Minutes
0
1
3
5
7
15
30
60
GAPDH
37kD
DAP12
25kD
DAP12
12kD

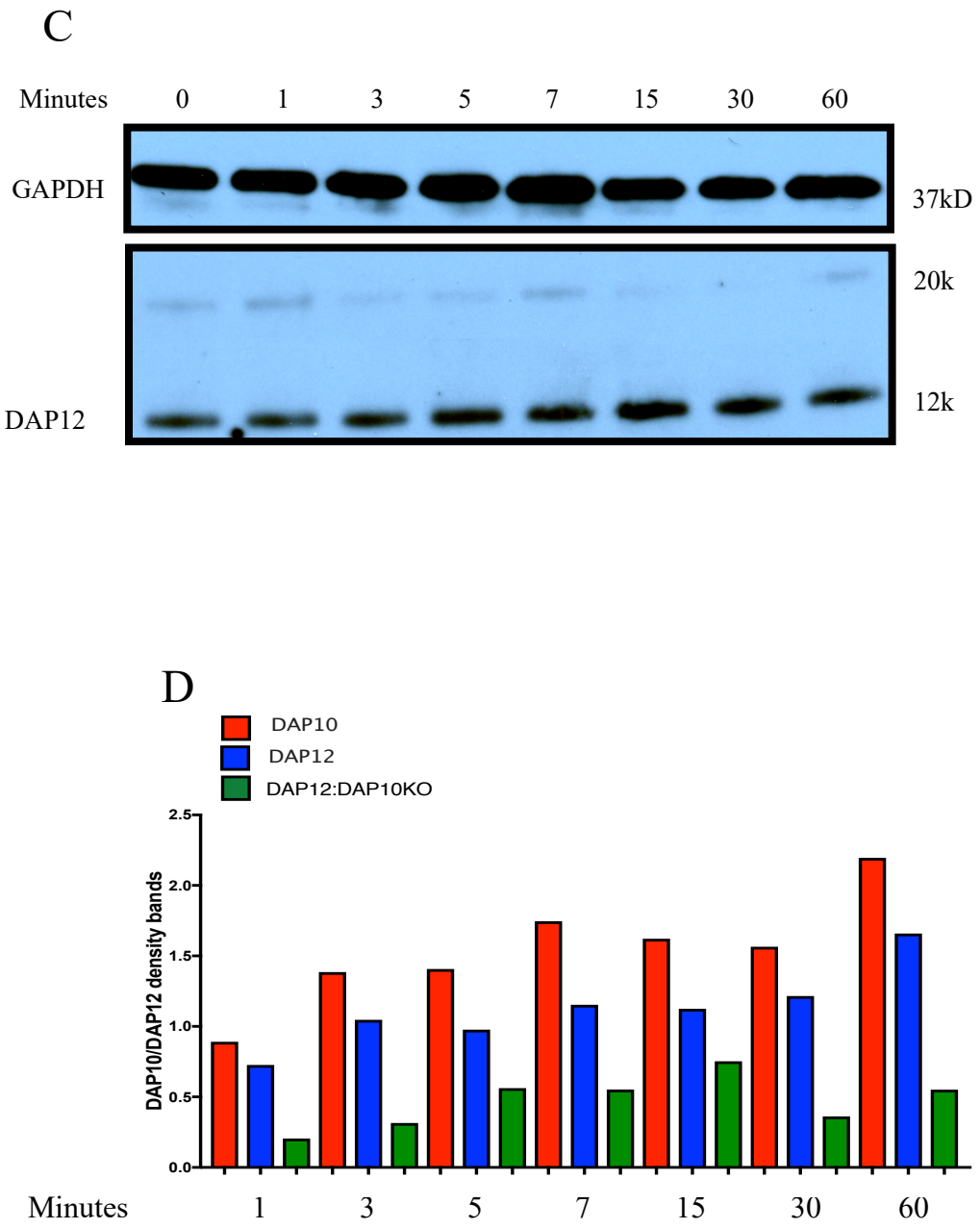


Figure 6. Dap10/Dap12 Form Complexes. CD11b cells isolated from (A&B) wildtype and (C) DAP10KO mice were pulsed with CD200AR-L P1 at various times and analyzed for DAP10 or

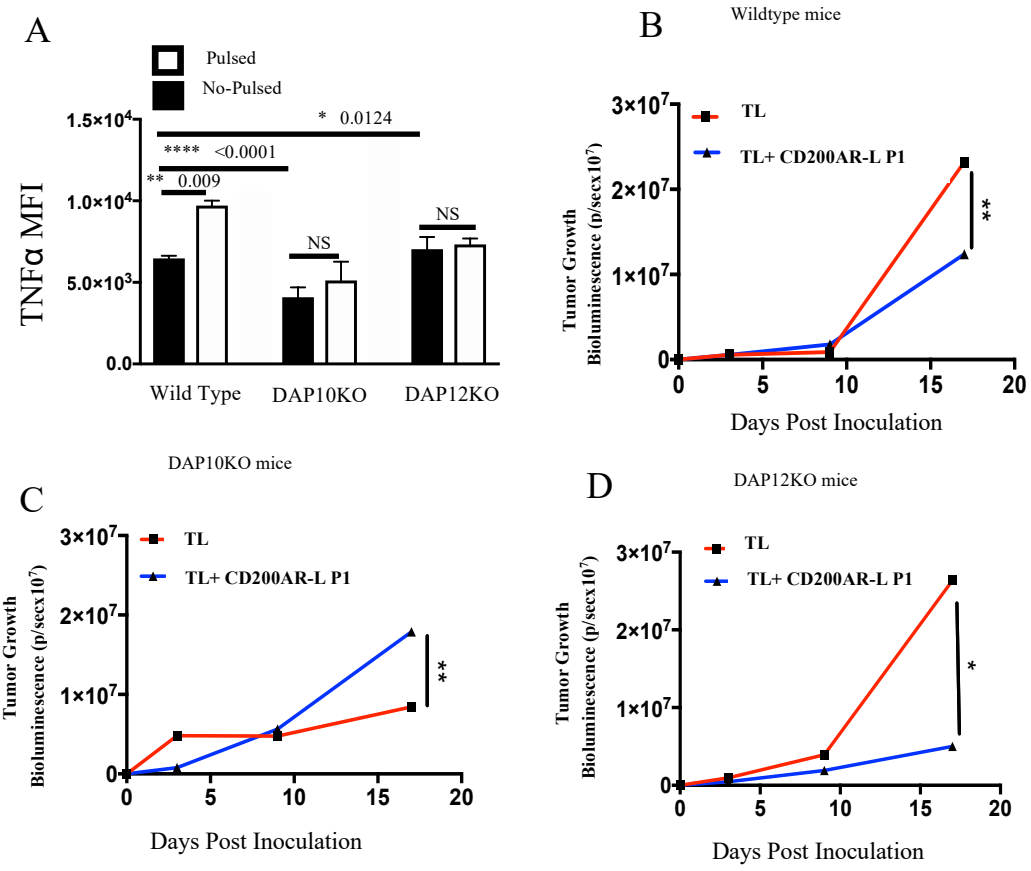
DAP12 protein levels. Density bands was measured to confirm the DAP10/DAP12 complex. Error bars are representative of standard deviation (2=3/group *P < 0.05 and **P<0.005; by t-test).

4.2.6 Knocking out the DAP10 and DAP12 Pathways Inhibits CD200AR-L Activation

To assess whether the presence of DAP10 or DAP12 are required for CD11b stimulation, CD11b cells were harvested from wildtype, DAP10KO and DAP12KO mice and pulsed with the CD200AR-L, supernatants were harvested and analyzed for TNF alpha production (Figure 7A). These findings validate our earlier studies demonstrating the CD200AR-L signals through DAP10 and DAP12 pathways. We observed that knocking out DAP10 and DAP12 inhibited the TNFα production.

Therefore, we next sought to determine if these studies translated *in vivo*. To accomplish this, wildtype, DAP10 and DAP12 knockout mice were given tumors and treated with tumor lysates (TL) or TL + CD200AR-L. We observed a significant decrease in tumor growth in wildtype mice inoculated with TL + CD200AR-L, however, in DAP10KO mice, we had a rapid increased in tumor growth in mice receiving TL+ CD200AR-L (Figures 7B&C). However, we observed decreased tumor growth in DAP12KO mice. Moreover, we observed that the removal of DAP10 significantly decreased survival in mice vaccinated with TL + CD200AR-L. These experiments determined that the DAP10 pathway is crucial to take control of the tumor growth by innate cells through the activation of CD200AR. We believe that DAP12 KO mice response to the CD200AR-L similarly to the wildtype mice because of the activation of DAP10 signaling pathways through the vaccination of CD200AR-L. Thus, these analyses based on both cell stimulation and *in vivo* study indicate that CD200ARs stimulation on

mouse CD11b couple to the signal-transducing subunit DAP10 to generate selective proximal signals that are fully capable of initiating CD11b activation.



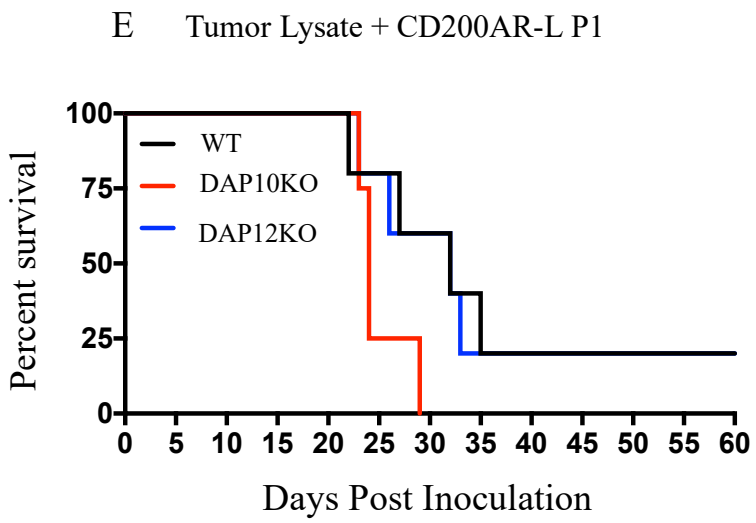


Figure 7. Knocking out the DAP10 and DAP12 Pathways Inhibit P1A12 Activation. (A) CD11b cells isolated from murine DAP10KO or DAP12KO or wildtype were pulsed with 10uM CD200AR-L for 48h, supernatant was collected and analyzed for TNFα production. To determine the effects of DAP10 and DAP12 *in vivo*, GL261 tumor bearing mice (wildtype (B), DAP10 (C), DAP12 KO (D)) were vaccinated with tumor lysates (TL) or TL + CD200AR-L and follow up for survival (E). Error bars are ± SEM, asterisks represent a statistical asterisk represent a statistical significance *$p < 0.05$, **$p < 0.05$ by T test or one-way ANOVA for *in vivo* studies.

4.3 Discussion

Our research focuses on the understudied CD200 immune checkpoint that modulates an immune response through paired receptors. Relatively, little is known of how CD200 exerts its effector immune functions particularly those regarding its activation receptors (Wright et al. 2003; Gorczynski et al. 2004; Voehringer et al. 2004; Hatherley et al 2005; Kojima et al. 2007; Lanier et al. 2009). This work shows that DAP10 couples to the CD200ARs stimulation and induces the downstream activation of phosphatidylinositol 3-kinase, Vav1, ERK1/2 and cJUN. Second, the signals initiated by

CD200ARs/DAP10 are fully capable of inducing cytokines secretion and controlling tumor growth. Third, the axis CD200AR-L P1/CD200ARs/DAP10 elicits proinflammatory cytokines, and chemokines, primarily (chapter 3), and these cytokines are crucial for the recruitment and trafficking of the immune cells, and the immune cells take control of the tumor. Thus, these findings identify a previously unknown mechanism by which receptor complexes of CD200ARs that lack ITAM motifs can trigger macrophage activation.

We pulsed CD11b cells isolated from wildtype (C57B6) mice and performed NanoString analysis, and data analysis was analyzed using IPA. These studies confirmed an upregulation of DAP10 and DAP12 molecules on cells pulsed with CD200AR-L. We next repeated the same experiment, but we analyzed the RNA for the DAP10 and DAP12 gene expression at different times. Interestingly, analyzing the time course determined that the DAP10/12 pathway increases within the 1st 5 minutes decreasing signaling after an hour, then the pathways are re-activated 24hrs post pulsing. Moreover, this data suggests that CD200AR-L stimulation induces the upregulation of DAP10 immune-related molecules, particularly, those sufficient to initiate phosphorylation of SLP-76 and Vav1 and MAPK kinases phosphorylation. These results explain our vaccination results. We determined that vaccinating mice with the peptide with tumor lysates enhanced survival, however, we determined that survival is significantly enhanced if we vaccinate the animals with the CD200AR-L then 24hrs later revaccinate with the CD200AR-L + tumor lysates (Moertel et al. 2014, Xiong et al. 2016, Olin et al. 2019). These experiments demonstrated the cell has a feedback loop re-stimulating the DAP10/12 pathways enhancing cell activation.

Although the DAP10 and DAP12 are independent pathways, they are thought to form complexes with each other to enhance and maintain signaling (Gilfillan et al. 2002). In response to ligation of TREM2, Src family kinases phosphorylate the ITAM of DAP12 and the YINM motif of DAP10, which form docking sites for the Syk and p85, with the subsequent recruitment of PLC-γ2 and Grb2 (Fig 1) (Tassi I, et al. 2006). We observed what we originally hypothesized as a dimerization of the DAP 10 and DAP12 bands in our western analysis. However, following a search in the literature, DAP10 and DAP12 forms cysteine bonds (Rabinovich et al. 2006). Therefore, we took DAP10KO CD11b cells pulsed with the CD200AR-L and analyzed them by western for DAP12. We observed the same DAP12 band at 12kD as observed in the wildtype cells, however, the band at 23 kD originally thought to be a dimer of the DAP12 validated that DAP10 and DAP12 bond together following activation. Hence, the CD200AR-L induces the activation of DAP10/DAP12 complex on antigen-presenting cells.

To further validate the role of DAP10 and DAP12 signaling pathways, we isolated CD11b cells from wildtype, DAP10 and DAP12 mice and pulsed them with the CD200AR-L. These experiments revealed a loss of function response to the CD200AR-L measured by TNFα production. Therefore, we wanted to determine if the benefit of using the CD200AR-L was lost in our tumor-bearing immunotherapy. We observed a significant reduction in wildtype mice and DAP12KO mice. However, we observed a significant increase in tumor growth in DAP10KO mice. These experiments demonstrated the importance of DAP10, although DAP12 is involved in CD200AR signaling, we suggest the ability to maintain a reduced tumor growth in DAP12KO mice was due to the DAP10 pathway. Moreover, we hypothesize the increased tumor growth

in DAP10KO mice is due to the ability to induce a cytolytic response. It is reported that human NKG2D-DAP10 triggers receptor for cytotoxicity in NK cells (Billadeau et al. 2003) and mediates primary stimulation signals, not only co-stimulatory signals, in NK cells (Lanier et al. 2019; Peng et al. 2010). Therefore, we lack the NK response capable of eliminating the tumor in DAP10KO mice. These studies correlate with our current studies showing that tumor-spontaneous-regression occur in CD200KO GL261 bearing-mice around 14d post-inoculation and have high NK infiltration (data not shown).

4.4 Materials and Methods

Animal housing: C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). DAP10 and DAP12-deficient mice were a generous gift from Dr. Lewis Lanier (University of California). Mice were housed in the specific pathogen-free animal facility at the University of Minnesota, according to institutional guidelines.

Brain inoculation: 8 to 16 week-old mice were anesthetized with an intraperitoneal injection of 0.03 ml ketamine HCl (100mg/mL)/xylazine (20mg/mL). For the stereotactic intracranial injection, the surgical site was shaved and prepared with 70% ethyl alcohol. Mice were placed in a stereotactic frame and a midline incision was made with a scalpel. A 1 mm burrhole was made 0.5 mm anterior to the bregma and 2.5 mm to the right of the midline. A Hamilton micro-syringe was inserted to a depth of 3 mm and withdrawn to a depth of 2.5 mm. 14×10^3 cells of GL261-Luc+ in 1ul saline was inoculated over four minutes. The needle was removed, and the skin was sutured with 4-0 nylon thread. Immediately after surgery, mice were injected with 5mg/Kg Carprofen and followed by

heating system. In addition, mice were administrated with Carprofen 5mg/Kg three days following tumor inoculation.

Vaccination schedule: Wildtype, DAP10KO or DAP12KO GL251-Luc^{+} mice (n=5) were vaccinated subcutaneously in back of the neck as described in figure 8. Mice received i) 65ug tumor lysate or ii) 65ug tumor lysate + 50 ug of P1, iii) saline treated group was used as a control.

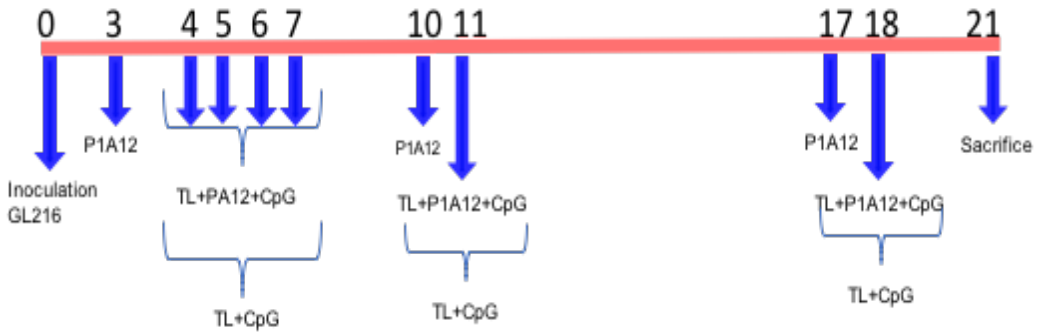


Figure 8. Vaccination Schedule

Bioluminescence Monitoring of Tumor Growth: Bioluminescence imaging was done weekly, beginning one week following tumor cell injection to monitor the tumor growth. Mice were anesthetized with 2% isoflurane, inoculated intraperitoneal with 100 mg/kg D-Luciferin potassium salt (BioVision) and imaged 10 minutes after injection. Bioluminescence was conducted using the IVIS 50 Lumina imaging station and regions of interest encompassing the intracranial area of signal/tumor, and the total

photons/second/steradian/square cm (photons/s/sr/cm2) were defined using living image software.

Cell Culture: GL261-Luc^{+} cells were grown in 25 ml of complete Dulbecco's Modification of Eagle's medium (DMEM), which consists of DMEM supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 1% nonessential amino acids in a T175 tissue culture flask incubating at 5% C02 and 37 °C. Prior to implantation the cultured cells are harvested by trypsinization, washed once in DMEM and resuspended in 1X PBS at a concentration of 15 x 10^3 cells/ul. Murine CD11b cells were isolated from DAP12, DAP10 KO and wildtype mice by using CD11b MicroBeads (Miltenyi Biotec). Wildtype and knockout macrophages (raw 264.7) were cultured in Roswell Park Memorial Institute- (RPMI-)1640 medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin.

Cytokine Secretion: Unless otherwise stated, all cells were treated at the same conditions for the cytokine secretion. A total of 1×10^6 cells was grown in 0. 500 ml in RPMI-1640 in a 48-well plate for 12 h, then pulsed with 10uM ligand P1, and incubated for an additional 48 h. LPS (1uM) and non-pulsed cells were used as controls. Supernatant (50ul) were collected and analyzed for TNFa levels using cytometry bead array (Biosciences). Data was analyzed using Flowjo v10.

Western analysis: 3×10^6 cells was grown in 0. 200 ml in RPMI-1640 in a 48-well plate for 12 hrs, then stimulated with 10uM ligand P1 for (0, 1, 3, 5, 7, 10, 15, 30, 60

min). Following stimulation, cells were lysed on ice for 10 min in 200 ul of RIPA buffer (Thermo Fisher Scientific). Equal amounts of lysate (20 ug) were loaded, resolved by 12% SDS-PAGE, transferred to Immobilon-P PVDF membrane (Millipore, Sigma), blocked with protein-Free T20 (TBS) blocking buffer (Thermo Fisher Scientific) and 2.5% milk, and probed with rabbit specific phoshpo antibodies table 2. All antibodies were used at a dilution of 1:1,000. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (HRP) (Jackson ImmunoResearch), and visualized with chemiluminescent substrate (Pierce Thermo Fisher Scientific). Non-pulsed cells were used as controls. Macrophages expressing two CD200ARs will be used to discriminate the DAP10 pathway mediated by CD200ARs.

Density measurements: Immunoblots were analyzed quantitatively by densitometry using an ibright fl1000 software (Thermo Fisher Scientific, Waltham, Massachusetts). Briefly, images were background-subtracted, and strips of the blot corresponding to each band were demarcated and analyzed for each time point/gel lane.

RNA extraction and quantitative PCR analysis: Naïve CD11b were plated at a density of 1×10^6 cells per 0.2 ml per well in 48-well in RPMI medium (described above) 1 d before the experiment and then stimulated with 10uM P1 and incubated for 1, 3, 5, 7, 15, 30, 60 min or 6h, 12h, 18h, 24h. RNA was extracted with Trizol according to the manufacturer's instruction. cDNA was synthetized with QUANTA kit according to the manufacturer's instruction. cDNA was analyzed by quantitative PCR with SYBR Green QPCR Mix on a QUANTA System. Primers were designed to amplify mRNA-specific

sequences corresponding the DAP10/12 molecules and, where possible, were placed on two separate exons spanning long intron in between. Gene expression was normalized to the expression of GAPDH or B-actin in the same sample and was calibrated to the expression in unstimulated wild-type samples. Inhibition assay. Naïve CD11b were treated as described above. Cells were pulsed with 10uM P38MAPK or 10nM Jak inhibitor for 1h before adding 10uM P1 and incubate for 6h. Then qPCR was set up as described above.

Chapter 5

Conclusion

5.1 Introduction

The goal of this dissertation was to elucidate the signaling pathway of the CD200 paired receptors addressing a major knowledge gap of the immune system. The information described in these research projects reveals the signaling pathway of the CD200 immune checkpoint that leads to activation, rather than suppression, of immune cells that can be used to improve the response of GBM to immunotherapy. The central hypothesis is that synthetic ligands modulate CD200 activation receptors overriding the inhibitory effect mediated by CD200 binding to CD200R demonstrating that ligands can serve as therapeutic agents for checkpoint inhibitor-based immunotherapy.

Towards testing our hypothesis, I developed and established CD200 receptor knockout macrophages from murine raw264.7 macrophages (Mϕs). I used the CRISPR/Cas9 system to generate different Mϕ cell lines expressing different combinations of or a single CD200 receptor. **The main achievements of this research are**: i] the DAP10 and DAP12 pathways are the immune activation signaling of the CD200ARs to activate antigen-presenting cells and enhance the immune response; ii] CD200 activation receptors operate in complexes in that a combination of two activation receptors are needed to elicit an immune response; iii] the suggested immune mechanism of CD200 and its receptors is mediated by the DAP10/PI3K/VAV1-ERK1/2/cJUN pathway; and iv] ligation of CD200ARs enhances survival in our murine glioma model through DAP10 signaling.

Until this research was completed, to our knowledge, there was no known information about CD200ARs on immune cells. This knowledge gap blocked the complete understanding of how the CD200 protein, and its receptors regulate the immune system and specifically myeloid lineage cells. This dissertation reveals for the first time that both the DAP10 and DAP12 pathways are involved in immune signaling pathways of CD200ARs. Hence, these data fill this knowledge gap and can be considered as ground-breaking research.

5.2 Overview of the Thesis

The first chapter of this thesis provides the underlying problem in treating GBM that lays the groundwork for this research. First, it noted the clinical relevance of glioblastoma and the failure of the current standard care providing reasons why it is crucial to develop new treatments. It highlighted the mechanisms by which GBM suppresses an immune response that hinders the efficacy of current immunotherapy. Moreover, it stated the importance of CD200 as an understudied immune checkpoint protein and how crucial it is in regulating an immune response through its paired receptors. Due the ability of the CD200 protein to modulate an immune response, it may be considered as a novel immune checkpoint inhibitor for therapy of brain tumors like GBM, as well as, peripheral tumors.

Furthermore, it has pointed out that the CD200 protein and its receptors are crucial to maintaining homeostasis of the immune system. The CD200 receptors are in the family of membrane-bound protein that can deliver inhibitory (through CD200R) or activating signals (through CD200ARs). It is known that the inhibitory signal is due to

specific binding of the native form of the CD200 protein to CD200R. CD200/CD200R binding induces activation of the canonical DOK pathway, recruits RasGAP and SHIP, and causes subsequent downstream inhibition of the RasMAPK pathways leading to suppression of immune cells.

One remarkable point is the lack of knowledge concerning CD200ARs. In spite of intensive investigation, ligands and immune signaling pathways had not been identified for CD200 activating receptors. There are two reports showing that two CD200 activation receptors, CD200AR4, and CD200AR3, coopt with the adaptor protein, DAP12, suggesting that these receptors would transmit strong activating signals. Nevertheless, the connection between recruitment of DAP12 and immune effector function had not been established.

Chapter 2 provides a review of published works of the Olin laboratory concerning establishing the utility of combining CD200-derived peptide with tumor lysate vaccines to treat GBM. The Olin laboratory has focused its attention on targeting an alternative immune checkpoint providing by CD200 immunosuppression. Our group is the first to promote two possible mechanisms of how CD200 immunosuppresses an antitumor response in GBM tumor microenvironment; i] CD200 is upregulated on the vascular endothelium of tumors leading to the immunosuppression of CD200R-bearing infiltrating leukocytes before they enter to the tumor microenvironment, and ii] CD200 is secreted from CNS tumors and interacts with the inhibitory CD200 receptor (CD200R) on immune cells in the tumor microenvironment and within the draining lymph nodes leading to suppression of anti-tumor response. We have demonstrated that the inhibitory effects of CD200 protein can be surmounted by selectively engaging CD200AR using

specific synthetic peptide ligands, CD200AR-Ls, that we identified using protein sequence analyses and structural data and have subsequently synthesized.

Finally, this laboratory has a great deal of experience in preclinical and clinical trials. We have demonstrated the efficacy of targeting the CD200ARs in immune activation, and survival in murine glioma models, where administration of the murine CD200-derivided ligand CD200AR-L in combination with tumor lysates significantly enhanced survival. Preclinical translational studies using a canine-specific CD200AR-L have resulted in a significant survival benefit in pet dogs with spontaneously developing high-grade glioma. In this study, 20 dogs were treated with canine-specific CD200AR-L and autologous tumor lysate vaccination following surgical resection of the gross tumor that provided a significant increase in median overall survival time compared to dogs treated similarly but without the CD200AR-L. These findings lead us to investigate further the activation effects of CD200ARs at the mechanistic level.

Chapter 3 describes the approach that I used to develop and test specific aim 1 and its correlate hypothesis. First, it describes how I developed and validated different cell lines expressing one, two or three CD200ARs using the CRISPR/Cas 9 system. Furthermore, it describes the immunofluorescence/cell-binding assay using knockout CD200ARs Mϕs to validate the binding of CD200AR-L on the cell-surface of Mϕs. Third, several cell stimulation assays were performed to assess the biological function of CD200ARs. It describes the research design, cell lines, instrumentation, data collection and analyses that were used in the study. These data show that the ligand, CD200AR-L, binds to CD200AR2 and CD200AR3 and Mϕs expressing complexes of CD200AR2/AR3 induces the greatest amount of TNFα production.

To summarize, the data in Chapter 3 provides evidence that CD200AR-L binds to CD200ARs cluster on the surface of antigen-presenting cells inducing immune activation demonstrated by cytokine secretion.

Chapter 4 presents the strategy used to uncover the activation signaling of CD200ARs. It describes protein activation at both, the phospho-activation and gene expression, levels. Several cell stimulation assays were performed to conclude that optimal DAP10 and DAP12 pathway activation occurs at 3 and 5 minutes, respectively. The main role of DAP10 on the CD200ARs signaling was demonstrated when TNFa secretion was abolished on pulsed DAP10KO CD11b cells with CD200AR-L.. Moreover, it includes data from *in vivo* experiments that validate the relationship between DAP10 and the CD200ARs. Overall, chapter 4 concludes that ligation between CD200AR-L and CD200ARs induces immune activation of APC through the DAP10 and DAP12 pathways.

5.3 Contributions of my Research

5.3.1 Contribution of Specific Aim 1

This research focused on the role of activation receptors (CD200ARs). This research shows that

- CD200ARs form complexes to exert their biological functions.
    - Macrophages expressing CD200AR2&3 and 3&4 responded to peptide stimulation by the CD200AR-L to increase TNF-α production.
    - Macrophages expressing a single CD200 receptor failed to induce TNF-α production.

    - Macrophages expressing CD200R1, 2&3 or 1,3&4 failed to bind the fluorescent CD200AR-L and failed to elicit TNFα production.
    - Macrophages expressing CD200AR2,3&4 bound fluorescent CD200AR-L but failed to produce TNFα.
- CD200AR2 may be the most important receptor in activating an immune response
    - Macrophages expressing CD200R1, 2&4 bind to fluorescent CD200AR-L and induce TNFα production.

Thus, these results show that innate cells, like macrophages, build distinct CD200AR complexes with different binding affinities to maintain homeostasis of the immune system. This is an important mechanism of how the immune system balances immune responses through receptor diversity.

5.3.2 Contribution of Specific Aim 2.

The main contributions to knowledge of the activation signaling of CD200ARs are:

- DAP10 and DAP12 pathways are the immune signaling of CD200ARs. DAP10 signaling induces cytolysis and cytokines secretion by natural killer cells when it associates with NKG2D. In our system, we found that monocytes (CD11b) activate DAP10/DAP12 pathways at different times after being pulsed with the ligand, CD200AR-L
- The ligand CD200AR-L activates a DAP10 cascade. DAP10 has a cytoplasmic activation motif that is a predicted binding site for the SH2 domain of the P85 subunit of PI 3-kinase (PI 3K). This work demonstrated that the CD200AR-L-

stimulation induces increased gene expression of the DAP10 related-molecules including PI 3K.

- DAP10/DAP12 form complexes. It has been demonstrated that DAP10 and DAP12 form heterodimers for signaling to induce activation of the immune system. These findings show that wildtype CD11b cells stimulated with CD200AR-L induce DAP10/DAP12 complex formation and no dimer formation was observed DAP10KO CD11b cells.
- Knocking out the DAP10 and DAP12 pathways inhibits the CD200AR-L activation. Stimulated DAP10KO CD11b cells with CD200AR-L fail to produce TNFα. However, both DAP12KO and wildtype CD11b cells produce TNFα when stimulated. Furthermore, *in vivo* experiments revealed that tumor-lysate and CD200AR-L treatment significantly increased tumor growth on DAP10KO mice-tumor-bearing. In contrast, wildtype and DAP12KO mice had significantly decreased tumor growth when treated with tumor lysate and CD200AR-L. These findings suggest that CD200AR-L induces DAP10 pathways, which is crucial to control of tumor growth. The reduced tumor growth in DAP12KO mice is likely due to the presence of the DAP10 pathway. Thus, we conclude that the CD200AR-L is crucial to activates DAP10 pathway on innate cells like macrophages -antigen-presenting cells- at the tumor microenvironment, so these cells take control of the tumor.

Finally, this work suggests that the CD200AR-L stimulation induces that DAP10 couples to the CD200ARs and stimulates downstream activation of phosphatidylinositol 3-kinase, Vav1, ERK1/2, and cJUN. These signals initiated by

CD200AR-L stimulation and CD200ARs/DAP10 activation are capable of inducing cytokine secretion in immune cells and controlling tumor growth in mice. Thus, these findings identify a previously unknown mechanism by which receptor complexes of CD200ARs that lack ITAM motifs can trigger macrophages activation.

5.4 Future Directions

Up to this time, little was known about the immunologically relevant expression of the CD200 receptors, except that CD200R is expressed in on some tumor cells. Further studies of the regulation of CD200ARs and their ligands may reveal the extent to which this system can provide protection against harmful conditions and whether any potentially detrimental effects are adequately counterbalanced by beneficial effects.

Another consideration is since CD200 and its receptors have been shown to be important in myeloid cell activation, elucidating the immunological contexts of how CD200ARs may induce functional processes involved in killing microbes, presentation of antigen to CD4+ or CD8+ T cells, and inflammation or tissue repair will lead to a more comprehensive understanding of how myeloid cells maintain the balance of the immune system.

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Appendices

Appendix 1

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Appendix 2

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Tumor-derived vaccines containing CD200 inhibit immune activation: implications for immunotherapy.

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Immunotherapy

Tumor-derived vaccines containing CD200 inhibit immune activation: implications for immunotherapy

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There are over 400 ongoing clinical trials using tumor-derived vaccines. This approach is especially attractive for many types of brain tumors, including glioblastoma, yet so far the clinical response is highly variable. One contributor to poor response is CD200, which acts as a checkpoint blockade, inducing immune tolerance. We demonstrate that, in response to vaccination, glioma-derived CD200 suppresses the anti-tumor immune response. In contrast, a CD200 peptide inhibitor that activates antigen-presenting cells overcomes immune tolerance. The addition of the CD200 inhibitor significantly increased leukocyte infiltration into the vaccine site, cytokine and chemokine production, and cytolytic activity. Our data therefore suggest that CD200 suppresses the immune system's response to vaccines, and that blocking CD200 could improve the efficacy of cancer immunotherapy.

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Keywords: CD200 protein • checkpoint blockade • immunotherapy

Despite four decades of intense research into vaccine-based strategies for fighting cancer, the majority of immunotherapies against solid tumors still fail to achieve beneficial outcomes. This is especially true for the CNS tumor glioblastoma multiforme (GBM). A recent search on ClinicalTrials.gov revealed over 400 open clinical trials using tumor cells as a source of antigens to stimulate an anti-tumor response; 25 of these are directed toward CNS tumors.

The use of tumors as a source of tumor-associated antigens clearly has advantages; however, most cancers have robust mechanisms for evading the immune system [1]. Immune checkpoint inhibitory ligands and their receptors tightly control T-cell activation, maintaining self-tolerance and limiting immune-mediated collateral tissue damage. Checkpoint blockades such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 receptor (PD-1) [2] have been targeted in multiple clinical trials, demonstrating some success (reviewed in [1]) [3]. We have extensively studied another checkpoint blockade (CD200/CD200R) responsible for shutting down the immune system [4,5] making the CD200 blockade interaction an important target for cancer immunotherapy [6–8].

CD200 has been well characterized as an immunosuppressive protein that inhibits immune responses through its receptor [9–11]. In healthy individuals, CD200 is distributed on a wide variety of tissues, including B cells, activated T cells, certain vascular endothelia, kidney, placenta cells and neurons [12]. In contrast to the distribution of CD200 ligand, its receptor, CD200 receptor (CD200R), is mainly expressed on myeloid cells (monocytes, granulocytes, dendritic cells). CD200R is also expressed on T cells and B cells, inactivating leukocytes through negative immune signals [13–15]. High expression of CD200R has also been detected on differentiated central and effector memory

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T cells. CD200R expression is particularly apparent in polarized Th2 cells [16], resulting in the expansion of regulatory T cells [17–19].

CD200 is expressed on tumors such as chronic lymphocytic leukemia [11], multiple myeloma [6], acute myeloid leukemia [20], melanoma [21], ovarian cancer [22], metastatic small cell carcinoma [23], GBM [4] and on the murine glioma GL261 (Figure 1A). In addition, tumor progression and poor patient outcome have been shown to correlate with the presence of soluble CD200 [24]. Wong *et al.* [24] reported that soluble CD200 levels in the plasma of chronic lymphocytic leukemia patients correlate with tumor burden and disease state. In our Phase I vaccine trial, we demonstrated increasing levels of CD200 in the serum of our GBM and ependymoma immunotherapy patients upon tumor recurrence [4].

Absorbing CD200 out of tumor-derived vaccines enhances immunogenicity

Because CD200 is expressed on tumors, we hypothesized that we are suppressing the immune system with the tumor-derived vaccines designed specifically to induce an anti-tumor immune response. To test our

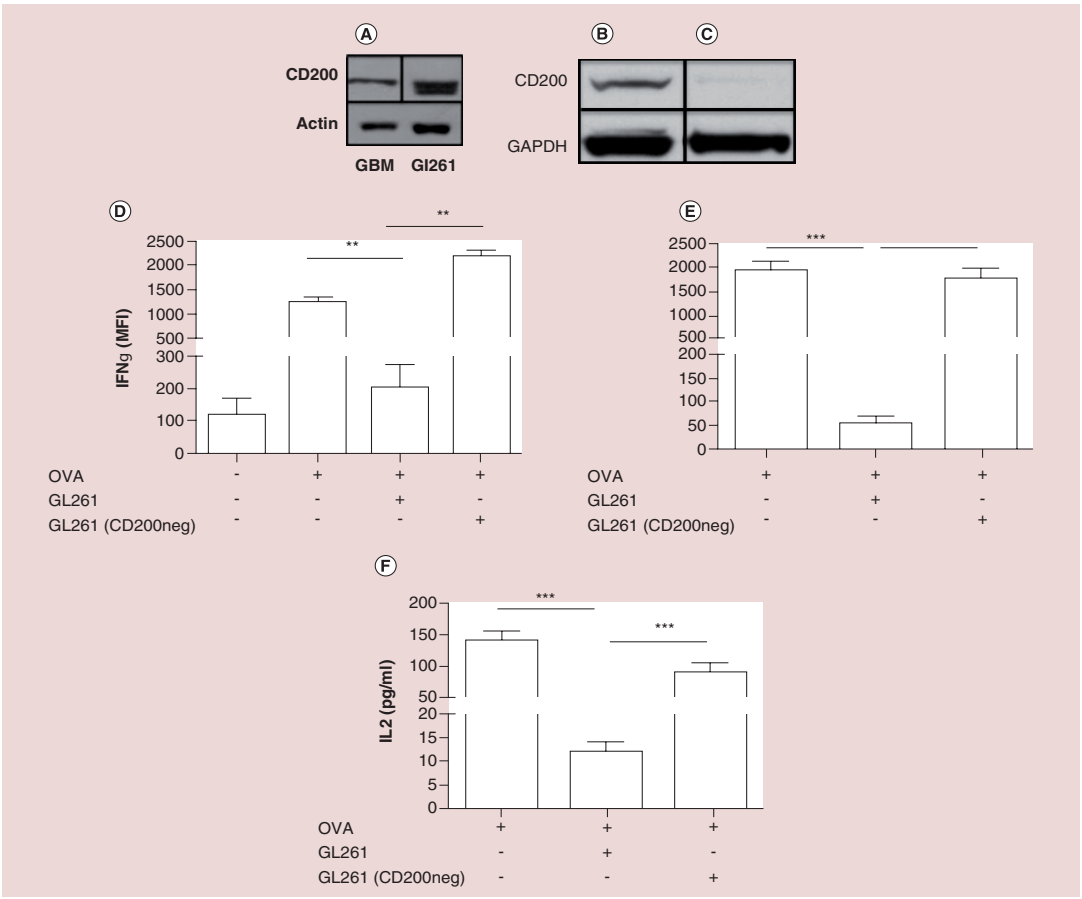


Figure 1. Absorbing CD200 out of tumor-derived vaccines enhances immunogenicity. (A) Human and mouse gliomas were analyzed by western analysis for CD200. **(B & C)** CD200 was absorbed out of murine GL261 tumor lysates and used to pulse **(D)** OT-1 splenocytes and **(E)** bone marrow derived dendritic cells with OVA as an immune stimulant with either wild-type GL261 or GL261 (CD200neg) tumor lysates. Error bars are ± SEM, asterisk represent a statistical significance *p < 0.05, **p = 0.005 or ***p = 0.0005 determined by unpaired t-test. Experiments are representative of three separate experiments.
OVA: Ovalbumin.

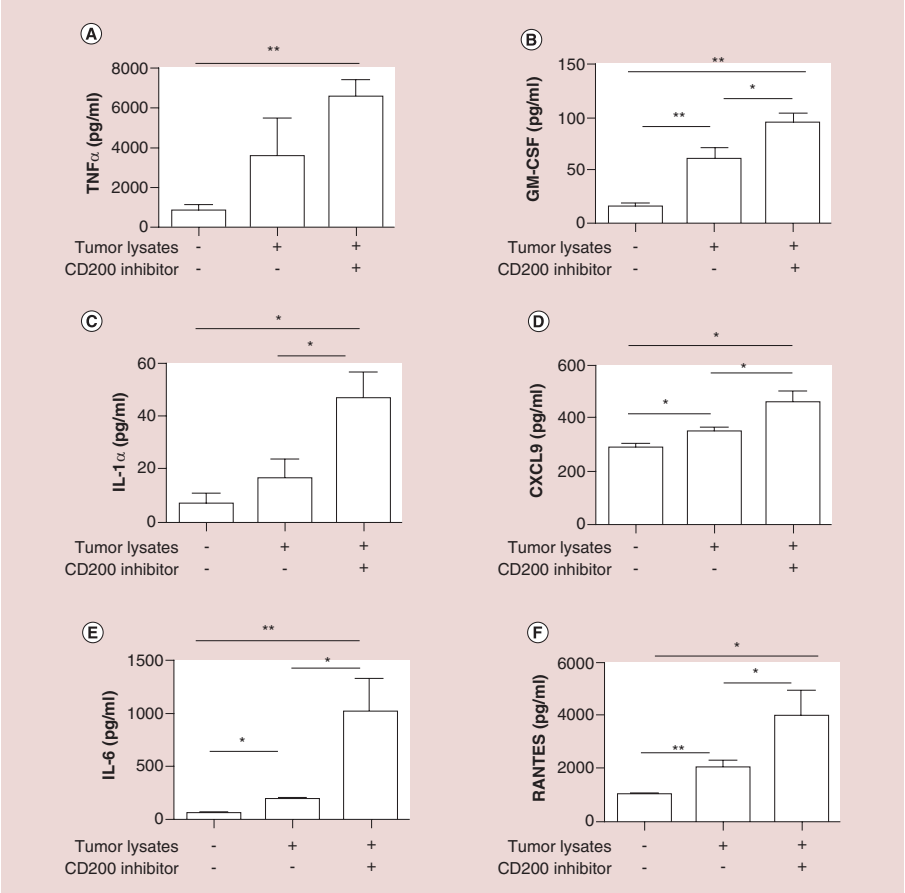


Figure 2. CD200 peptide inhibitor blocks the suppressive properties of CD200. (A–F) CD11b cells were isolated from C57BL/6 wild-type mice were pulsed with tumor lysates derived from wild-type Gl261 cells +/- the CD200 peptide inhibitor. Supernatants were analyzed for chemokine and cytokine secretion. Error bars are ± SEM, asterisk represent a statistical significance *p < 0.05 or **p = 0.005 determined by unpaired t-test. Experiments are representative of three separate experiments.

hypothesis, we depleted CD200 from our tumor lysates using immunoprecipitation (Figure 1B & C). OT-I splenocytes were pulsed with ovalbumin (OVA) + GL261 tumor lysate (GL261) or GL261 depleted of CD200 (GL261 (CD200neg)). GL261 significantly suppressed the ability of OVA to induce an immune response (p = 0.009), which was reverted by depleting CD200 from the vaccine (p = 0.003) (Figure 1D). Because CD200 acts on antigen-presenting cells [19], we repeated this experiment with bone marrow-derived dendritic cells (DCs). Our experiments recapitulated the findings in Figure 1D that, compared with OVA alone, tumor lysates containing CD200 inhibited IFN-γ (p = 0.001) and IL-2 (p = 0.005) production, a result which was reversed by depleting CD200 (p = 0.001) (IFN-γ), p = 0.001 (Figure 1E) and (IL-2) (Figure 1F).

CD200 inhibitor blocks immune suppression from tumor-derived vaccines

Targeting receptor–ligand interactions has become increasingly important, as indicated by CD200/CD200 receptor (CD200R) in leukemia cells and CD47/SIRP in many cancers cells [11,25–27]. We developed a peptide inhibitor targeting the CD200R isoform activation receptors [4]. Purified CD11b cells from wild-type mice were pulsed with tumor lysate contain-

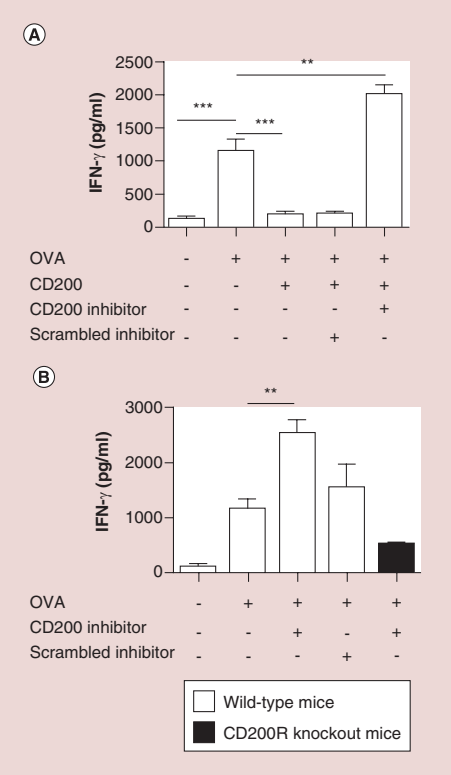


Figure 3. CD200 inhibitor enhances an antigen-specific response. (A & B) Bone marrow-derived dendritic cells from wild-type C57Bl/6 or CD200R KO mice were pulsed with OVA, OVA + CD200, OVA + CD200 + CD200 inhibitor or OVA + CD200 + scrambled inhibitor. Following 24 h incubation, cells were washed, and purified OT-I CD8 T cells were added. Following 48 h incubation, supernatants were analyzed for IFN-γ production. Experiments are representative of three separate experiments. Error bars are ± SEM, asterisks represent a statistical significance *p < 0.05, **p = 0.005 or ***p = 0.0005 determined by unpaired t-test.

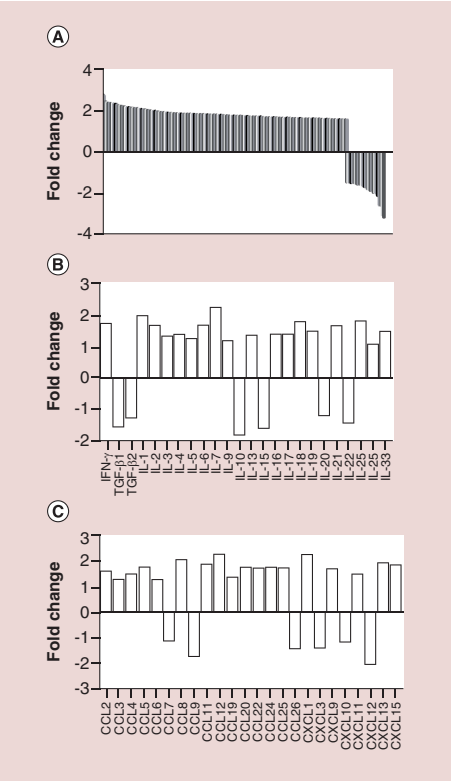


Figure 4. CD200 inhibitor modifies gene expression. Purified CD11b cells isolated from wild-type C5Bl/6 mice were pulsed with CD200 inhibitor. RNA was isolated and analyzed by NanoString for 575 immune-related genes. Bars represent a **(A)** ± twofold change or **(B & C)** ± 1.5-fold change.

ing CD200, with or without the CD200 inhibitor. In these experiments, with the exception of TNF-α and IL1α (p = 0.07 and p = 0.12 respectively), tumor lysates alone elicited a statistically significant cytokine response (p = 0.003 (GM-CSF), p = 0.012 (IL6), p = 0.02 (CXCL9) and p = 0.006 (RANTES) compared with no pulse controls. The CD200 inhibitor treatment group achieved a statistically significant enhanced immune response p = 0.004 (TNF-α), p = 0.001 (GM-CSF), p = 0.033 (IL1α), p = 0.015 (CXCL9), p = 0.001 (IL6) and p = 0.013 (RANTES) compared with no pulse control and p = 0.015 (GM-CSF), p = 0.023 (IL1α), p = 0.015 (CXCL9), p = 0.015 (IL6) and p = 0.046 (RANTES) compared with tumor lysate groups alone (Figures 2A–F). We observed enhanced secretion of TNF-α when adding the CD200 inhibitor to tumor lysates, however, these results failed to reach statistical significance (p = 0.069).

CD200 inhibitor enhances an antigen-specific response

To generate a tumor-specific immune response, CD8 T cells undergo priming by DC, the antigen-presenting cell most efficient at initiating potent $CD8^+$ T-cell responses [28,29]. Currently, the efficacy of *ex vivo* derived DC immunotherapy is not well established for human cancers [30–33]. The limited success of these immunotherapies has been attributed to a variety of factors, including the preparation and administration

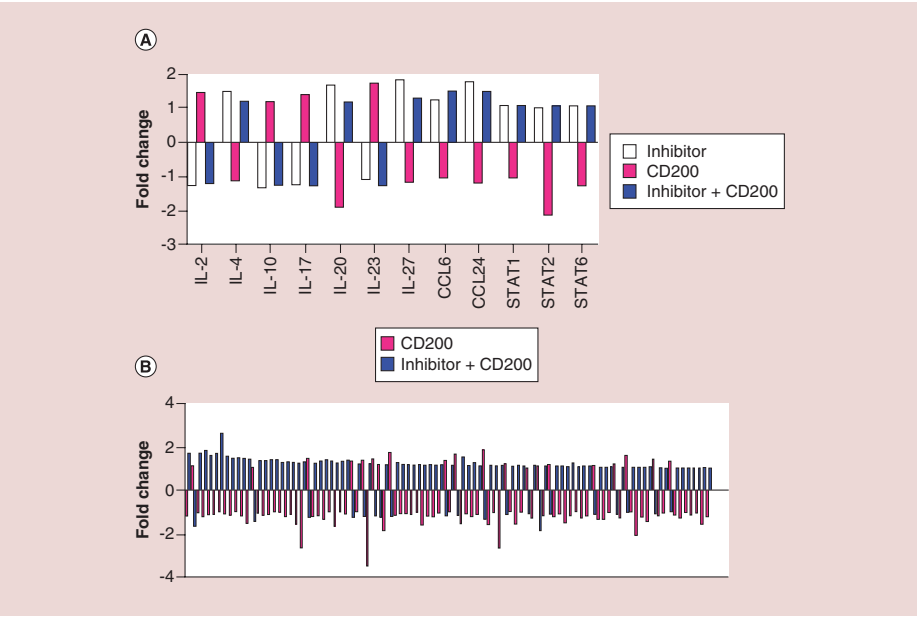


Figure 5. CD200 inhibitor reverses CD200 protein inhibitory signals. Purified CD11b cells isolated from wild-type C5Bl/6 mice were pulsed with a CD200 protein, CD200 inhibitor or a combination of CD200 protein and CD200 inhibitor. RNA was isolated and analyzed by NanoString for 575 immune-related genes. Bars represent a ± 1.5-fold change.

of the vaccine, the disease stage of the participants in experimental trials, or the heterogeneous nature of most tumors. We suggest the failure to elicit an anti-tumor response is due to CD200 in tumor-derived vaccines used to activate DC.

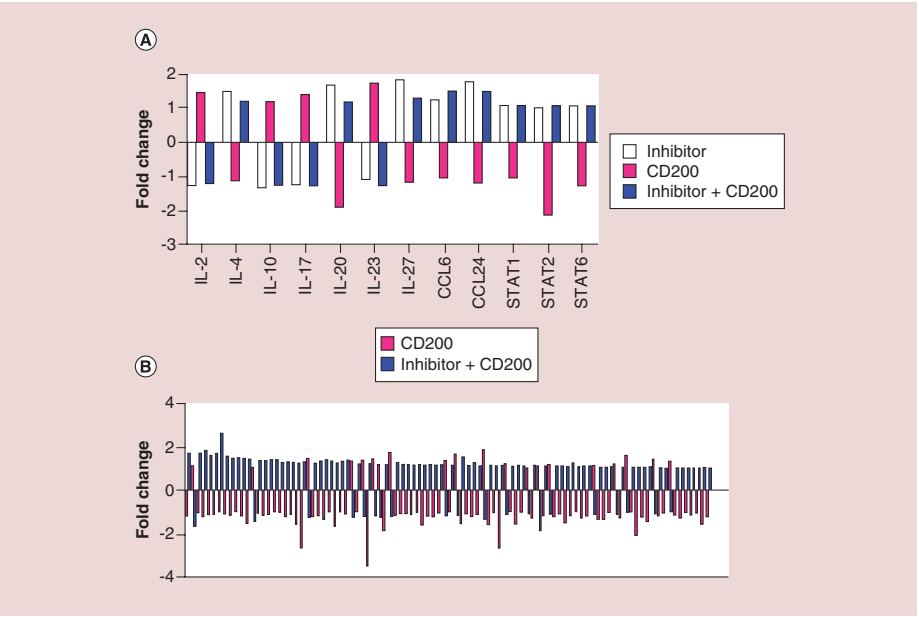
To test this, bone marrow-derived DC from wild-type mice were pulsed with OVA + CD200 with or without the CD200 inhibitor. Following 24 h incubation, cells were washed to remove any free inhibitor, then incubated with purified OT-I cells. As previously demonstrated *in vivo* [4], the CD200 inhibitor blocked the suppressive effects of CD200, reverting to an antigen-specific OVA immune response (Figure 3A). OVA significantly enhanced an IFN-γ response ($p = 0.007$), which was suppressed with the addition of CD200 ($p = 0.009$). The addition of the CD200 inhibitor overpowered the suppressive properties of the CD200 protein, significantly enhancing an immune response ($p = 0.003$), as compared with using OVA alone. Interestingly, in these experiments, we observed that cells pulsed with CD200 inhibitor + OVA significantly enhanced the immune response ($p = 0.001$) (Figure 3B) compared with OVA treated cells. These studies led us to hypothesize that the CD200 inhibitor activates antigen-presenting cells.

CD200 inhibitor modifies gene expression

To test our hypothesis that the CD200 inhibitor activates antigen-presenting cells, CD11b cells from wild-type splenocytes were pulsed with CD200 protein, CD200 inhibitor or a combination of CD200 protein + CD200 inhibitor and analyzed by NanoString for 575 immune-related genes. All treatment groups were normalized to no pulse controls. In these experiments, 194 immune-related genes had a ± 1.5-fold change following pulsing with the CD200 inhibitor alone (Figure 4A–C).

When we compared all three treatment groups, we observed that 98 genes within the CD200 protein group had an opposite response compared with genes within the CD200 inhibitor or CD200 protein + CD200 inhibitor treatment groups (Figure 5A & B). These experiments demonstrated that the CD200 inhibitor reversed the inhibitory signaling induced by the CD200 protein.

To determine if the CD200 inhibitor activated functional responses, DCs were pulsed with the CD200 inhibitor alone. These experiments revealed that the CD200 inhibitor activated DCs, statistically enhancing the production of IL-2, TNF-α, IL-1α, IL-6, GM-CSF and IL-1β ($p = 0.01$, $p = 0.02$, $p = 0.04$, $p = 0.001$,



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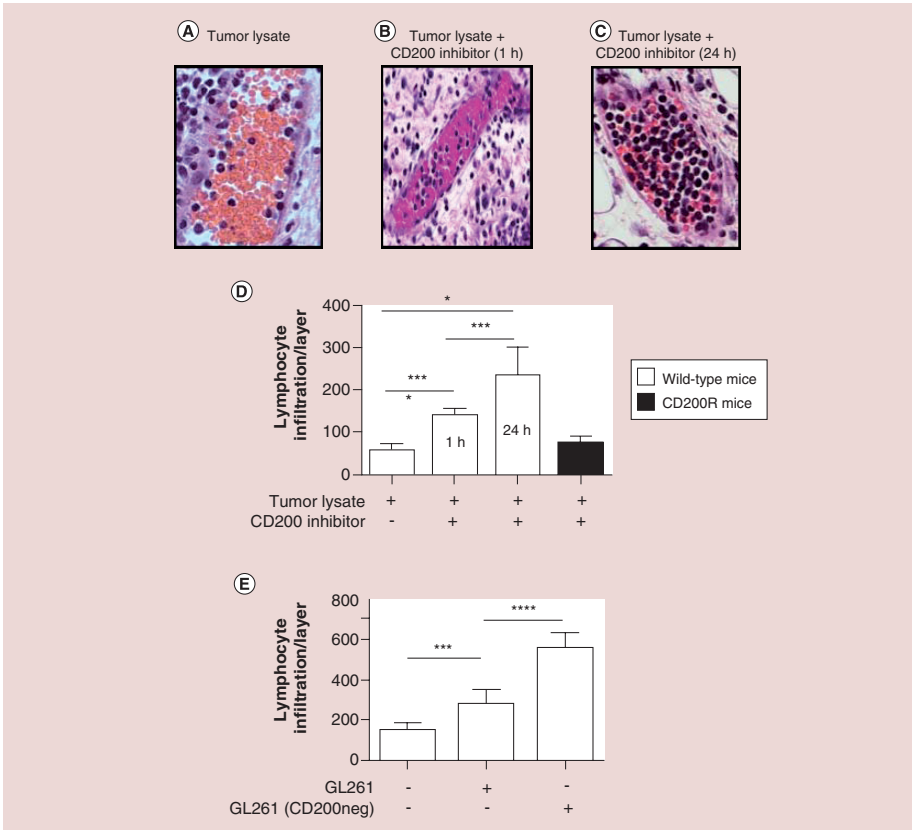


Figure 7. CD200 inhibitor enhances leukocyte trafficking into the vaccine site. Non-tumor-bearing C57Bl/6 or CD200R knockout mice were vaccinated with tumor lysates or CD200 inhibitor, either 1 or 24 h later, mice were revaccinated with **(A)** tumor lysates + CpG or **(B)** tumor lysates, CD200 inhibitor + CpG (1 h revaccination). **(C)** tumor lysates, CD200 inhibitor + CpG (24-h revaccination). 6 h later, skin from the vaccine sites was harvested and analyzed by H&E staining. **(D)** Leukocytes within blood vessels in eight separate skin levels were counted. **(E)** In separate experiments, mice were vaccinated with wild-type GL261 lysates or GL261 lysates void of CD200. 24 h later, mice were revaccinated with either wild-type GL261 lysates or GL261 lysates void of CD200 + CpG. 6 h later, skin was harvested and leukocytes within blood vessels in eight separate skin levels were counted. Error bars are ± SEM, asterisks represent a statistical significance *p < 0.05, **p = 0.005 and ***p = 0.0005 determined by unpaired t-test.

with the CD200 inhibitor 1 h prior to revaccination with tumor lysates + CD200 inhibitor (Figure 7A–C).

6 h following revaccination, skin at the vaccine site was harvested and analyzed for leukocyte infiltration. No significant leukocyte infiltration was observed in saline vaccinated controls or in CD200R KO mice vaccinated with tumor lysates + CD200 inhibitor (data not shown). To quantify our results, vascular leukocytes from eight layers of tissue were counted (Figure 7D). These experiments demonstrated enhanced leukocyte infiltration into the vaccine site with as little as 1-h pre-vaccination with the CD200 inhibitor ($p = 0.001$; 1 h and $p = 0.001$ 24 h) (Figure 7D). Moreover, knocking out the CD200 receptor failed to enhance leukocyte infiltration ($p = 0.087$).

These experiments demonstrated that while we were capable of eliciting an immune response using tumor-derived vaccines, the response failed to recruit antigen-presenting cells to the site of vaccination for antigen uptake. We next wanted to see how removing CD200 from tumor lysate vaccines influenced leukocyte infiltration. In these experiments, non-tumor-bearing wild-type mice were vaccinated with tumor lysate or tumor lysate void of CD200. 24 h later, mice

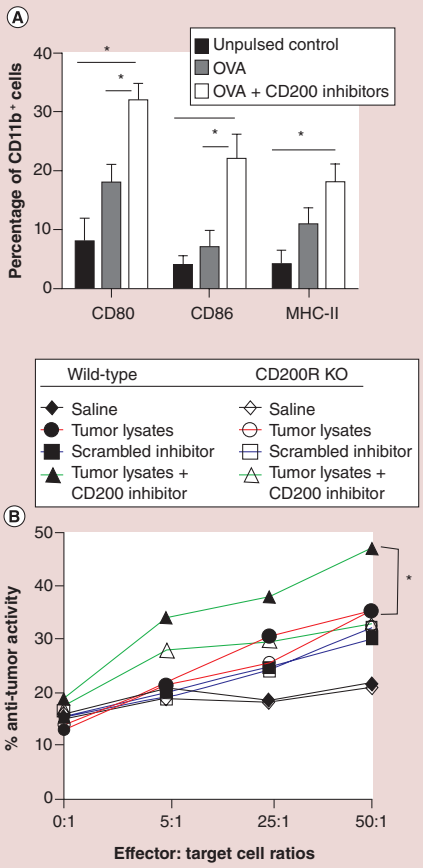


Figure 8. CD200 inhibitor enhances an antitumor response. (A) Purified CD11b cells from wild-type C57Bl/6 cells were pulsed with OVA ± CD200 inhibitor. Forty-eight hours later, cells were analyzed for CD80/86 and MHC-II expression. **(B)** Tumor-bearing wild-type (solid symbols) or CD200 receptor knockout (CD200R KO) (open symbols) mice were vaccinated in the back of the neck with saline (black lines), wild-type GL261 tumor lysates (red lines), tumor lysates + scrambled CD200 inhibitor (blue lines) or tumor lysate + CD200 inhibitor (green lines). 20 days post vaccination, lymphocytes from cervical lymph nodes were harvested, incubated for 6 h with wild-type GL261 cells and analyzed for cytolytic activity. Asterisks represent statistical significance *p < 0.05 determined by two-way ANOVA.

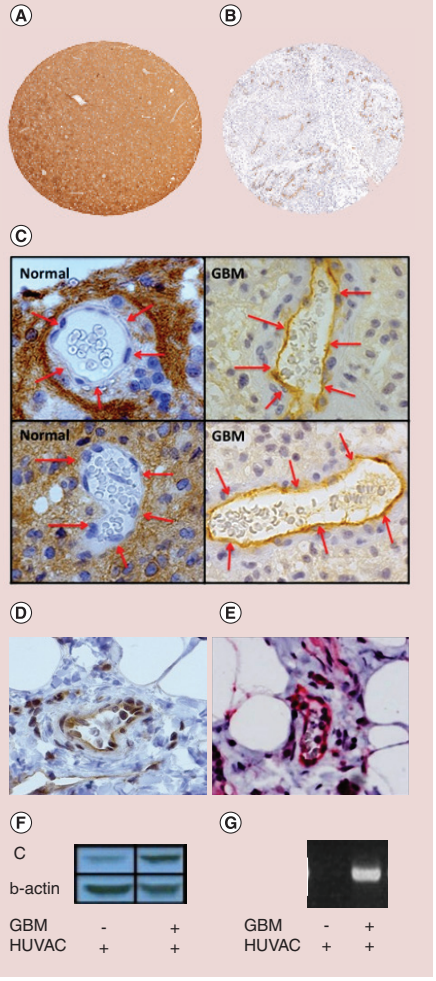
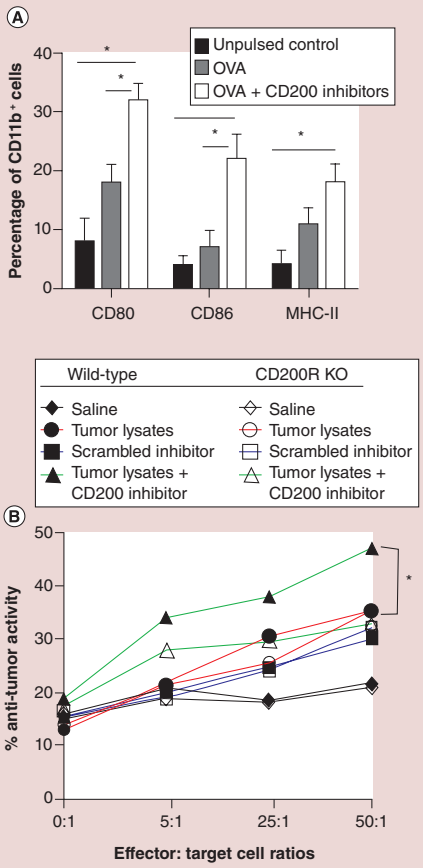


Figure 9. CD200 is upregulated in vascular endothelial cells. Tissues isolated from **(A)** normal human CNS or **(B)** glioblastoma multiforme (GBM) were analyzed for CD200 expression. **(C)** Vascular endothelial cells from normal tissue and GBM **(D)** breast tumor and **(E)** melanoma cells were analyzed for CD200 expression. Human endothelial cells were expanded on the bottom of a trans-well plate. GBM cells were placed on the top of the plates and incubated for 48 h. HUVAC were washed and analyzed by **(F)** western analysis and **(G)** RT-PCR for CD200 transcription.

were revaccinated with tumor lysate + CpG-ODN or tumor lysate void of CD200 + CpG-ODN, respectively (Figure 7E). As seen in the above experiments, we observed a significant infiltration of leukocytes into the site of vaccination ($p = 0.004$), however, removal of CD200 profoundly enhanced leukocyte infiltration ($p = 0.0001$) (Figure 7E).



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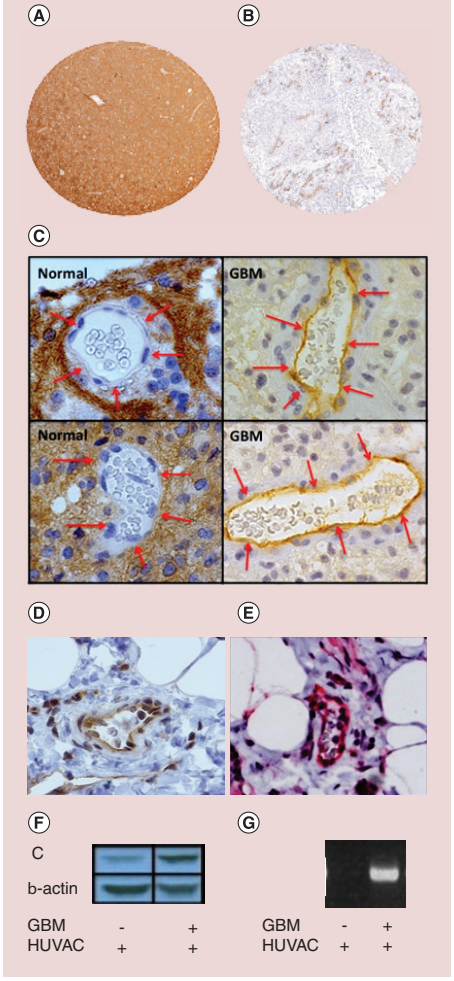

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et al. [35]. Mice were vaccinated with tumor lysates with or without the CD200 inhibitor (Figure 8B). Scrambled inhibitor was used as a control. Twenty days post inoculation, lymphocytes from draining cervical lymph nodes were harvested and incubated with GL261 cells to initiate a tumoricidal response. Two-way ANOVA revealed a statistically significant enhancement of an anti-tumor response by lymphocytes with the addition of the CD200 inhibitor ($p = 0.001$) (Figure 8B).

Individual analysis by Student's t-test revealed that tumor lysates in both wild-type and CD200R knockout mice with significantly enhanced anti-tumor responses ($p = 0.001$ and $p = 0.001$) at effector:target cell ratios of 25:1 and 50:1, respectively, as compared with the saline treatment group. In addition, the CD200 inhibitor group significantly enhanced anti-tumor responses ($p = 0.001$, $p = 0.001$ and $p = 0.001$) at effector:target cell ratios of 5:1, 25:1 and 50:1, respectively, as compared with the saline treatment group. In wild-type mice, the CD200 inhibitor treatment group exhibited significantly enhanced antitumor responses at effector:target cell ratios of 5:1, 25:1 and 50:1 ($p = 0.0001$, $p = 0.026$ and $p = 0.003$, respectively) as compared with the tumor lysate treatment group. In addition, there was a significantly enhanced anti-tumor response between CD200 inhibitor and CD200 scrambled inhibitor control treatment groups in wild-type mice at effector:target cell ratios of 5:1, 25:1 and 50:1 ($p = 0.001$, $p = 0.0066$ and $p = 0.0018$, respectively). We also observed significantly enhanced anti-tumor responses at effector:target cell ratios 5:1, 25:1 and 50:1 ($p = 0.006$, $p = 0.016$ and $p = 0.006$, respectively) between wild-type and CD200R KO mice in the CD200 inhibitor treatment group. No significant differences were observed between wild-type and CD200R KO mice treated with tumor lysates or the scrambled inhibitor. These experiments demonstrated the ability of our inhibitor to enhance an anti-tumor response when used in conjunction with a tumor-derived vaccines.

CD200 is upregulated on endothelial cells

Inhibiting CD200/CD200R interactions has been suggested as a method to enhance immunotherapy [11,36–39]. A clinical trial sponsored by Alexin Pharmaceuticals (NCT00648739) developed a monoclonal anti-CD200 (ALXN6000) to block tumor-derived CD200 expressed on B-cell chronic lymphocytic leukemia and multiple myeloma cells from interacting with $CD200R^+$ lymphocytes (clinicalTrials.gov) [39]. No results have been posted in clinicaltrials.gov. We do not anticipate that this method will be a very efficacious therapy. Twito *et al.* [40] has demonstrated that 'A Disintegrin And Metalloprotease' enzyme (ADAM28) sheds CD200 from B-cell chronic lymphocytic leukemia [40], which would invalidate the use of an antibody to block tumor-driven CD200–CD200R interactions. Our preliminary data correlate with Twito's findings. We reported high transcription levels of CD200 in GBM [4], however, staining for CD200 protein revealed that, in contrast to normal CNS, GBM have low CD200 expression (Figure 9A & B) potentially due to secretion.

To validate CD200 protein expression on GBMs, human GBM were analyzed for CD200 expression by western analysis. In contrast to normal CNS tissue, there was low expression of CD200 on the tumors. However, closer examination revealed that GBMs increase expression of CD200 on endothelial cells within the blood–brain barrier (Figure 9C). The same CD200 expression was seen in the vasculature of human breast carcinoma (Figure 9D) and melanoma (Figure 9E). To determine the ability of GBM to upregulate CD200, human endothelial cells (HUVAC) were placed on the bottom of a trans-well plate and human GBM was placed on the top. Following 72-h incubation, HUVAC cells were harvested and analyzed by western immunoblot (Figure 9F) and RT-PCR (Figure 9G) for CD200. These experiments demonstrated that GBM induces $CD200^+$ endothelial cells.

Conclusion

CD200 has been well described as immunosuppressive, making it a logical target for immunotherapy [4,8,11]. We have been extensively interrogating the multiple mechanisms by which CD200 inhibits the development of an antitumor response. We suggest that the CD200 in the tumor-derived vaccines and the CD200 protein secreted from the tumor micro-environment will inhibit the ability of antigen-presenting cells to mount an antitumor response (Figure 10A & B). We also argue that our CD200 peptide inhibitor, through the activation of a CD200 isoform receptor, reverses CD200-induced suppression (Figure 10C).

Our model is supported by studies reporting that CD200/CD200R interactions have been characterized as inhibitory receptors [26,41]. CD200R contains tyrosine motifs which signal through the recruitment of DOC2 to distinguish the CD200R from almost all other inhibitory receptors that have immunoreceptor tyrosine-based inhibition motifs [42]. However, additional CD200R-like proteins have recently been identified in mice and humans [13]. Four separate CD200 receptor genes have been identified [13]: CD200R1,

CD200R2, CD200R3 and CD200R4 [41,43]. These receptors are predicted to be associated with DNAX activating protein, (DAP)12, known to potentiate and attenuate activation of leukocytes [13,44]. Although the CD200R isoforms have not been well characterized, Gorczynski *et al*. reported that specific peptide sequences within the CD200 protein act as antagonists. Gorczynski hypothesizes that these peptide sequences bind to one of the CD200R isoforms that normally contribute an activation signal [45].

Our data correlate with Gorczynski's hypothesis. We suggest that our CD200 inhibitor is targeting one of the activation isoforms of the CD200 receptor. However, CD200 has multiple mechanisms of inducing immune suppression. Following close examination of CD200 immunohistochemistry, we have demonstrated that CD200 is upregulated on vascular endothelial cells (Figure 9C). $CD200^+$ endothelial cells appear to be tumor-specific because the surrounding CNS does not express CD200 in the blood–brain barrier vasculature (data not shown). This is an important discovery because others have reported that tumor-CD200 expression differentiates $CD4^+CD200R^+$ cells into a suppressor T-regulatory population (reviewed in [46]) [17]. We suggest that CD200R-bearing leukocytes will interact with $CD200^+$ endothelial cells to differentiate $CD4^+CD200R^+$ to regulatory T cells, leading to the development of an immunosuppressive tumor environment (Figure 10C).

Future perspective

Breaking CD200/CD200R interactions intensifies the success of antitumor therapy (reviewed in [46]). We developed a 13 amino acid CD200 peptide inhibitor that, given with tumor lysate, significantly enhances immunogenicity in our glioma model, as well as our breast carcinoma model [4]. We are now focusing our efforts on a mechanism to overcome the suppressive $CD200^+$ endothelial cells (Figure 9C). We are developing a monoclonal anti-CD200R specific for the same epitope as our CD200 inhibitor, which we hope will block the differentiation of immune suppressor cells. We hypothesize that, following T-cell activation, systemic inoculation of the anti-CD200R will bind the CD200R on $CD200R^+$ leukocytes. Our preliminary data suggest that blocking CD200R will allow CD200R leukocytes to enter the tumor microenvironment, escaping differentiation into their suppressive populations.

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Financial & competing interests disclosure

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Executive summary

Tumor-derived CD200 in vaccines inhibits an anti-tumor response

- Tumor-derived vaccines are widely used for solid tumor immunotherapy.
- Tumor-derived vaccines contain immunosuppressive proteins.
- CD200/CD200R interaction is an immune checkpoint manipulated by tumors and suppressing an immune response, enhancing immune escape.

CD200 inhibitor blocks immune suppression from tumor-derived vaccines

- CD200 peptide inhibitor blocks the suppressive effects of CD200 in tumor-derived vaccines.
- CD200 inhibitor enhances leukocyte infiltration into the vaccination site.

CD200 inhibitor enhances immunogenicity

- Tumor lysate combined with the CD200 inhibitor significantly enhances the development of an anti-tumor response.

CD200 inhibitor activates antigen-presenting cells

- CD200 inhibitor acts as an agonist activating antigen-presenting cells, enhancing immune activation.

CD200 is upregulated on vascular endothelial blood vessel cells

- Glioblastoma multiforme, breast tumors and melanoma upregulate CD200 on endothelial cells surrounding tumors, enhancing immune escape.

Conclusion

- CD200 is a major limitation for the development of an anti-tumor response.
- CD200 peptide inhibitor may be used to enhance solid tumor immunotherapy.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Appendix 3

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CD200 Checkpoint Reversal: A Novel Approach to Immunotherapy

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CD200 Checkpoint Reversal: A Novel Approach to Immunotherapy

Zhengming Xiong[1], Elisabet Ampudia Mesias[1], G. Elizabeth Pluhar[2,3], Susan K. Rathe[2], David A. Largaespada[1,2], Yuk Y. Sham[4,5], Christopher L. Moertel[1,2], and Michael R. Olin[1,2]

ABSTRACT

Purpose: Advances in immunotherapy have revolutionized care for some patients with cancer. However, current checkpoint inhibitors are associated with significant toxicity and yield poor responses for patients with central nervous system tumors, calling into question whether cancer immunotherapy can be applied to glioblastoma multiforme. We determined that targeting the CD200 activation receptors (CD200AR) of the CD200 checkpoint with a peptide inhibitor (CD200AR-L) overcomes tumor-induced immunosuppression. We have shown the clinical efficacy of the CD200AR-L in a trial in companion dogs with spontaneous high-grade glioma. Addition of the peptide to autologous tumor lysate vaccines significantly increased the median overall survival to 12.7 months relative to tumor lysate vaccines alone, 6.36 months.

Experimental Design: This study was developed to elucidate the mechanism of the CD200ARs and develop a humanized peptide inhibitor. We developed macrophage cell lines with each of four CD200ARs knocked out to determine their binding specificity and functional response. Using proteomics, we developed humanized CD200AR-L to explore their effects on cytokine/chemokine response, dendritic cell maturation and CMV pp65 antigen response in human $CD14^+$ cells. GMP-grade peptide was further validated for activity.

Results: We demonstrated that the CD200AR-L specifically targets a CD200AR complex. Moreover, we developed and validated a humanized CD200AR-L for inducing chemokine response, stimulating immature dendritic cell differentiation and significantly enhanced an antigen-specific response, and determined that the use of the CD200AR-L downregulated the expression of CD200 inhibitory and PD-1 receptors.

Conclusions: These results support consideration of a CD200AR-L as a novel platform for immunotherapy against multiple cancers including glioblastoma multiforme.

Introduction

The discovery of immune checkpoints and their inhibition ("checkpoint blockade") is a recently developed modality for the treatment of cancer that has truly revolutionized care for some patients (1, 2). Current FDA-approved checkpoint inhibitors are mAbs that can extend survival in patients with selected solid tumors such as melanoma. However, many solid tumors respond poorly to checkpoint inhibitors. This includes glioblastoma multiforme (GBM; ref. 3), an incurable primary central nervous system (CNS) tumor with a median overall survival of 14.6 months with the current standard of care (4, 5). Combinations of inhibitors to target multiple immune checkpoint pathways have been employed in an effort to significantly enhance survival. Unfortunately, these combinations can cause severe immune-related adverse events, often leading to treatment discontinuation or morbidity and mortality (6–9).

The CD200 immune checkpoint causes suppression of the secretion of proinflammatory cytokines, including IL2 and IFNγ (10, 11), and increases production of myeloid-derived suppressor cells (12) and T regulatory cells (12–14) resulting in compromised antitumor activity. Previously, we discovered the following mechanisms employed by the CD200 protein for immunosuppression: (i) it is upregulated in GBM-associated endothelial cells creating an immunologic barrier around the tumor microenvironment (10); and (ii) it is shed from tumors (12, 15) and interacts with the CD200 inhibitory receptor (CD200R1) on immune cells both in the tumor microenvironment and draining lymph nodes (10, 15). Our research focuses on the development of a therapeutic agent that targets the CD200 immune checkpoint regulatory system, which is known to modulate an immune response through CD200R1 (10, 12).

However, in addition to the inhibitory CD200R1, there is a series of activation receptors (CD200AR2, 3, 4, and 5) in mice (16). By using specific synthetic peptide ligands (CD200AR-L) that we identified through protein sequencing and structural analyses of CD200 (10), we developed a peptide-based strategy to engage these activation receptors on immune cells (10) and demonstrated that the inhibitory effects of CD200 protein can be surmounted by selectively engaging CD200ARs (11, 17). This was accomplished using specific CD200AR-L that we identified through protein sequencing and structural analyses of the native CD200 protein (10). The ability to overpower the suppressive effects of CD200 is lost when using a scrambled CD200AR-L or CD200ARKO mice, demonstrating that these peptides mimic active sites within the CD200 protein to modulate CD200AR activity resulting in immune stimulation (10, 12).

We tested the efficacy of the CD200AR-L in companion dogs with spontaneous high-grade glioma using a canine-specific peptide (18). In this study, intradermal injections of the canine CD200AR-L prior to and during administration of autologous tumor lysate vaccines

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

This report evaluates the ability to modulate the CD200 immune checkpoint by employing synthetic peptides directed as ligands to its paired immune activation receptor. We previously reported the presence of CD200 in the sera and tumor vasculature of patients with glioblastoma multiforme (GBM). We have also shown that a canine CD200AR-L extends the lives of companion dogs with high-grade glioma. The data we present here show that the human CD200AR-L directed to the CD200 activation receptor on $CD14^+$ cells activates immune upregulation through induction of cytokine response and dendritic cell differentiation. In addition, hCD200AR-L downregulates the inhibitory CD200 and PD-1 receptors. These findings provide a basis to evaluate hCD200AR-L as a novel immune therapy for patients with GBM. Downregulation of PD-1 suggests that hCD200ARL may also obviate the need for PD1- and PD-L1–directed therapies for GBM and other malignancies.

significantly enhanced the efficacy of this immunotherapeutic modality, doubling the median overall survival time compared with dogs receiving tumor lysate vaccines alone (18). We found the therapeutic effect of CD200AR-L compelling enough to translate these findings into the human clinical setting. Herein, we describe the binding of CD200AR-L to specific CD200ARs on antigen-presenting cells (APC) resulting in immune activation. We also describe the development of specific human CD200AR-Ls that enhance the ability of human APCs to initiate an antigen-specific response.

Materials and Methods

Transfection

Cells from the macrophage cell line, Raw 264.7, were incubated in RPMI1640 supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C until confluent. Upon confluency, transfection was performed using the Neon electroporation system (Thermo Fisher Scientific). A total of 5×10^4 cells were harvested and incubated in 10-mL Neon Buffer R with 1 μL (1 μg/μL) of Clean-Cap Cas9 mRNA (TriLink Biotechnologies) and 1-μL (100 pmol/μL) CRISPR evolution sgRNA Synthego (Synthego) for 2 minutes. Following incubation, cells were placed in a Neo electroporator at 1,720 pulse voltage, 10 pulse width, and two pulse numbers. Two days after transfection, cells were analyzed by PCR to validate the deletion of each CD200AR.

Immunofluorescence cell-binding assay

A total of 5×10^4 macrophages were grown in a Lab-Tek II 8 chamber slide in 200-mL RPMI containing 10% calf serum and 1% penicillin/streptomycin. At approximately 70% confluency, cells were washed twice with 1× PBS, pulsed with 10 μmol/L biotinylated CD200AR-L for an hour, fixed in 4% paraformaldehyde for 20 minutes at room temperature, then incubated with streptavidin Alexa Fluor 568 conjugate (Thermo Fisher Scientific) for 1 hour, washed with 1× PBS, and stained with 1-μg DAPI. Imaging was performed using an Inverted Ti-E Deconvolution Microscope System (Nikon Instruments Inc.).

Peptide synthesis

Human peptides (P1: IVTWQKKKAVSPENM, P2: NITLEDEGCYMCLFN, P3: VTFSENHGVVIQPAY and P4: CLFNTFGFGKISGTA) were synthesized (Thermo Fisher Scientific; **Fig. 2A**). The purity of the peptides was >95% and each peptide was modified by N-terminal acetylation and C-terminal amidation to enhance their stability.

Cytokine measurements

A total of 5×10^5 human $CD14^+$ cells were isolated from peripheral blood mononuclear cells (PBMC) using anti-CD14 beads (BD Biosciences) with a typical yield of ≥70% recovery and ≥90% purity. Cells were pulsed with 2 μmol/L of each CD200AR-L, P1, 2, 3, or 4 and incubated for 48 hours. The supernatants were then analyzed by bead array for cytokine production (BD Biosciences).

Dendritic cell differentiation

$CD14^+$ cells were purified from cytomegalovirus positive (CMV^+) $HLA\text{-}A2^+$ lymphocyte packs (American Red Cross) as described above. Approximately 8×10^8 cells were cultured in polystyrene tissue culture flasks at 37°C in 5% CO_2. GM-CSF (25 ng/mL) and IL4 (40 ng/mL) were added on days 3 and 5 to derive immature dendritic cells (iDC).

Cytomegalovirus assay

iDCs (5×10^5) were pulsed with 10-μg cytomegalovirus (CMV) antigen peptide $pp65_{495–503}$ (NLVPMVATV) and cultured as described above. iDCs were washed three times and coincubated with $CD8^+$ T cells from CMV^+ donors (5×10^5). PBMCs from CMV^- donors were used as a negative control. Supernatants were collected after 48 hours of incubation and analyzed for IFNγ production by cytometric bead array (BD Biosciences).

NanoString gene expression analysis

Total RNA from $CD14^+$ cells was sent to New Zealand Genomics Limited to measure the expression of genes that are differentially expressed during inflammation (nCounter GX, NanoString Technologies). Briefly, total RNA was extracted from $CD14^+$ cells (MagJET RNA kit, Thermo Fisher Scientific) using the protocol adapted for tissue (KingFisher Duo machine, Thermo Fisher Scientific). RNA samples were then quantified (Qubit 2.0 fluorometer, Thermo Fisher Scientific) and subjected to RNA integrity analysis (2100 Bioanalyzer, Agilent Technologies). Probes for the genes encoding CD44 (NM_001001392.1), NANOG (NM_024865.2), OCT4 (NM_002701.4), STAT3 (NM_139276.2), and the housekeeping genes glucuronidase beta (GUSB; NM_000181.1), clathrin heavy chain (CLTC; NM_4859.2), and hypoxanthine phosphoribosyltransferase 1 (NM_000194.1) were designed and manufactured by NanoString Technologies.

Expression data obtained with NanoString GX were analyzed using nSolver Analysis Software 3.0 (nanostring.com/products/nSolver) using default settings and normalized to housekeeping genes. nSolver performed cluster analysis and generated heatmaps using Java Treeview Version: 1.1.6r4. Pathway analysis was performed using PathCards Pathway Unification Database (pathcards.genecards.org; ref. 19). Student *t* tests were used to determine significant differences among groups ($P < 0.05$).

Results

Murine CD200AR-L binds CD200AR2&3 and CD200AR3&4 to activate APCs

The CD200 checkpoint modulates immune responses through paired receptors; an inhibitory receptor (CD200R1; ref. 20) and several activation receptors (CD200ARs). Two CD200ARs are expressed on human immune cells and four on murine cells (CD200AR2–5;

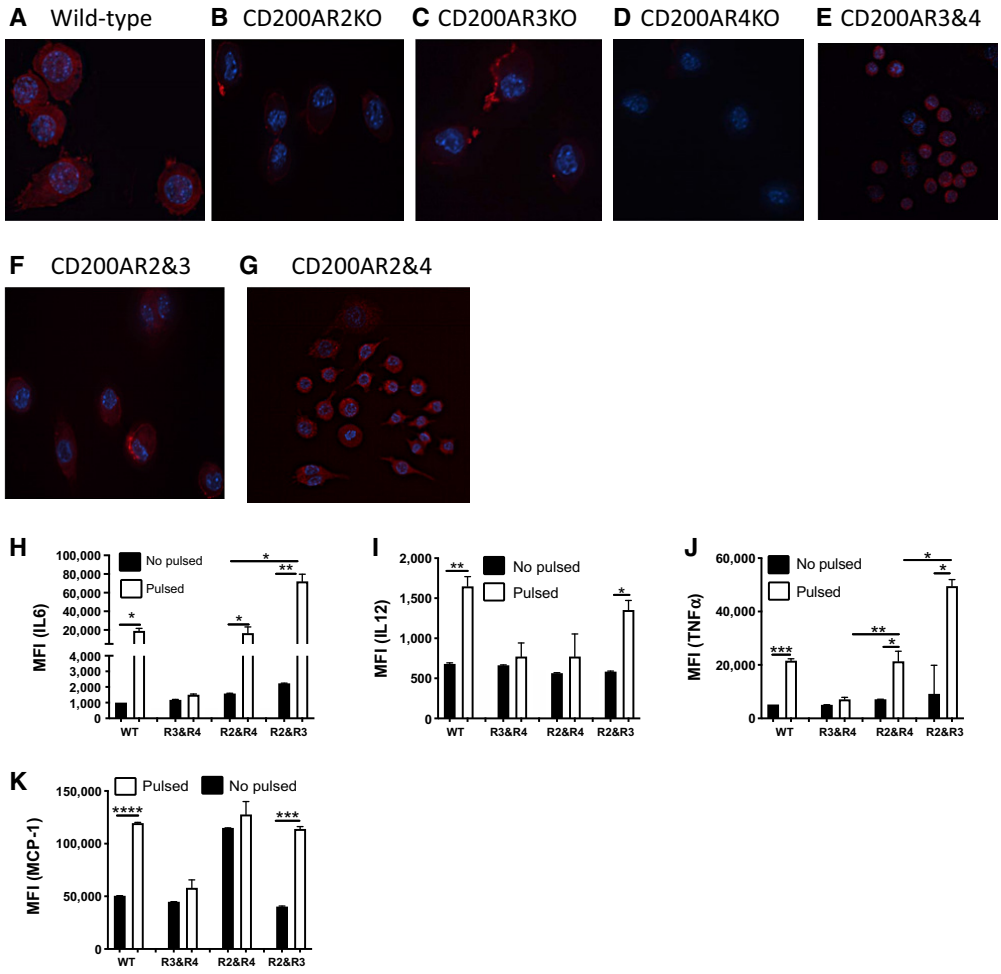

Figure 1.
CD200AR-L binds to CD200AR complexes. Wild-type (**A**), CD200AR2KO (**B**), CD200AR3KO (**C**), CD200AR4KO (**D**), CD200AR3&4 expressing (**E**), CD200AR2&3 expressing (**F**), and CD200AR2&4–expressing macrophages (**G**) were pulsed with fluorescently labeled CD200AR-L and assessed by microscopy. Cells were pulsed with the unlabeled CD200AR-L, incubated for 48 hours, and supernatants were analyzed for alterations in IL6 (**H**), IL12 (**I**), TNFα (**J**), and MCP-1 production (**K**). Nonpulsed cells with the same receptors were used as controls. Error bars, SD ($n = 3$/group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$ by t test).

refs. 11, 21). Although interactions between CD200 and the inhibitory receptor have been characterized, the natural ligands for the activation receptors and the molecular signaling that results from ligation remain unknown. We have demonstrated that targeting CD200ARs may represent a promising approach for immunotherapy by enhancing an antiglioma response in induced murine and spontaneous canine models with the addition of CD200AR-L to autologous tumor lysate vaccination. However, we wished to establish a better understanding of the mechanisms involved in targeting CD200ARs before translation to patients with human glioblastoma. To achieve this, a murine macrophage cell line was pulsed with a fluorescently labeled murine CD200AR-L (**Fig. 1A**) to validate binding. Using CRISPR, we created macrophage cell lines expressing single or combinations of CD200ARs. To achieve this, macrophage cells first had either CD200R1 or one of the CD200ARs knocked out (Supplementary Fig. S1A). These single knockout cell lines were then used to develop dual receptor knockout cell lines, which then had further receptor knockouts to create cell lines expressing a single CD200 receptor (Supplementary Figs. S1B–S1F). Cells were validated by PCR to validate gene removal using wild-type cells as a positive control. All

receptor knockouts were sequenced to validate gene removal (Supplementary Figs. S1A, S1G, and S1H). Cells with receptor 1, 3, and 4 knockout were validated by flow cytometry; however, there is no commercially available anti-CD200AR2 antibody. Relative to wild-type cells, reduced peptide binding was seen on CD200AR2KO and CD200AR3KO and no peptide binding was observed on CD200AR4KO cells. However, we subsequently demonstrated strong peptide binding on cells expressing CD200AR2&3, CD200AR3&4, and CD200AR2&4 (**Fig. 1A–G**).

We next wanted to determine the functional effects of ligand binding to the different CD200ARs. Cell lines were pulsed with the murine CD200AR-L and supernatants were analyzed for cytokine production. These experiments correlated with the binding experiments in that the pulsed cells expressing CD200ARs 2&3 and 2&4 had a significant increase in IL6 and TNFα production, and those expressing CD200AR2&3 had a significant increase in IL12 and MCP-1 compared with the unpulsed controls (**Fig. 1H–K**). These experiments demonstrated that the CD200AR-L targets activating receptor complexes, specifically CD200AR2&3 and CD200AR3&4, to activate APCs.

Design and identification of inhibitory peptides against CD200AR-L

Because we were now confident that we were targeting activation receptors with the peptide ligands, we sought to develop human-specific CD200AR-Ls for clinical use. Previous analysis of regions of CD200 that interact with CD200ARs revealed four regions with significant homology among the human, canine, and murine proteins (**Fig. 2A**). Four CD200AR-L peptides termed P1–4 were generated. To determine whether these peptides activated human APCs as we previously observed for the murine CD200-mimic peptides (10), purified human $CD14^+$ cells were pulsed with each of the four CD200AR-L peptides and supernatants were analyzed for immunostimulatory cytokines. We observed a significant increase in IL1β ($P = 0.0126$, $P = 0.0364$, $P = 0.0022$, $P = 0.008$) and TNFα ($P = 0.0146$, $P = 0.0007$, $P = 0.0002$, and $P = 0.0082$) in $CD14^+$ cells pulsed with P1, P2, P3, or P4, respectively, compared with unpulsed controls (**Fig. 2B** and **C**). To determine an antigen-specific response, we used a CMV model in which T cells from CMV^+ donors are primed with the CMV antigen pp65. Pulsing iDCs with CMV antigen pp65 and each of the CD200AR-L peptides elicited a significant antigen-specific response exemplified by IFNγ production ($P = 0.034$, $P = 0.033$, $P = 0.0042$ and $P = 0.020$; P1-4 respectively) compared with pulsing with pp65 alone (**Fig. 2D**).

We next conducted an alanine scanning experiment that is designed to identify the specific amino acid residues responsible for the peptide's conformation, stability, and function. Alanine is sequentially substituted for each nonalanine residue in each of the four peptides. The corresponding change in epitope activity was measured to identify the peptide that induced maximal immune stimulation of APCs. Sixty-one

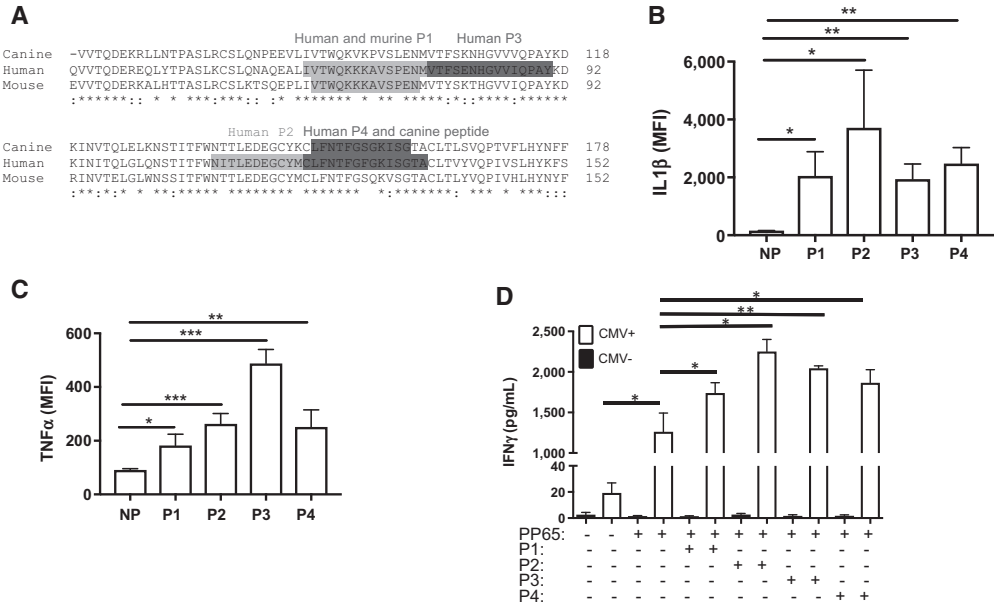

Figure 2.

Targeting CD200ARs stimulates APCs. **A,** Amino acid sequences of canine, human, and murine CD200 protein showing the homology of the various CD200 peptides. $CD14^+$ cells were pulsed with peptides 1–4 and incubated for 48 hours. Nonpulsed cells were used as a control. Supernatants were harvested and analyzed for IL1β (**B**) and TNFα (**C**). **D**, iDCs were pulsed with the CMV antigen pp65 ± peptides 1–4. Cells were washed and autologous T cells were added and incubated for another 48 hours. Supernatants were harvested and analyzed for IFNγ production. Error bars, SD ($n = 3$/group; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test between treatment groups). Data from cells of three separate healthy donors.

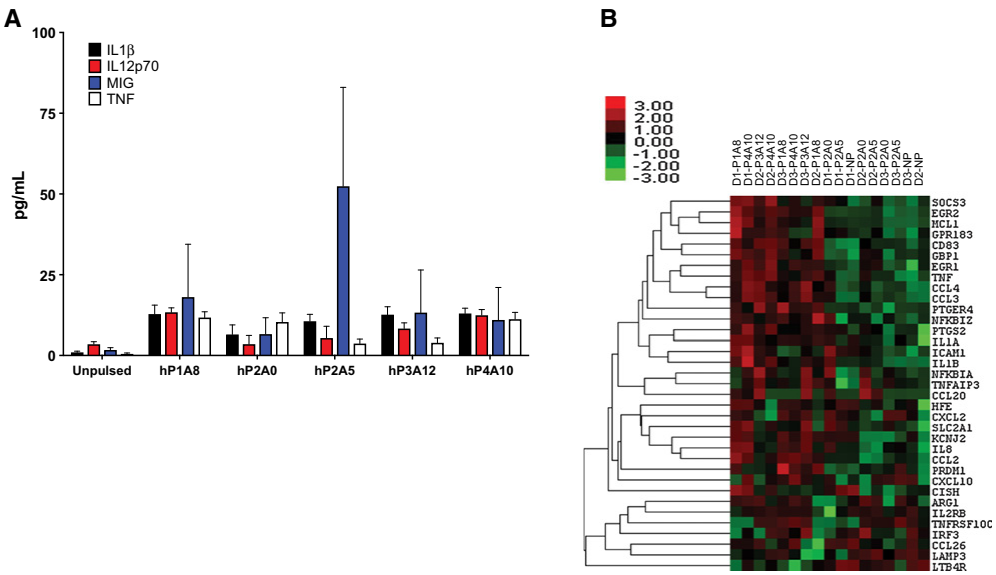

Figure 3.

Alanine substitutions enhance antigen-presenting cell stimulation. **A,** $CD14^+$ cells were pulsed with equimolar ratio of peptides, P1A8, P2A0, P2A5, P3A12, and P4A10, and incubated for 48 hours. Supernatants were harvested and analyzed for IFN1β, IL12p70, MIG, and TNFα production. **B,** $CD14^+$ cells were incubated with each peptide for 1 hour and then RNA was harvested and analyzed by NanoSight for alterations in immune-related transcripts. Pulsed cells were normalized to a nonpulsed (NP) control to derive a heatmap by nSolver using Java Treeview. Cluster analysis of the 35 genes that showed significant expression changes in one or more of the treated samples when compared to the NP controls (excludes the outlier D1-P3A12). Error bars, SD ($n = 3$ donors run in triplicate).

Table 1. Pulsed and nonpulsed CD14 cells were analyzed by IPA analysis for upregulation of the TNF pathway.

Pathways	Gene upregulation
LDL oxidation in atherogenesis	*CCL2, CCL3, ICAM1, IL1B, TNF*
Immune response, MIF-mediated glucocorticoid regulation	*ICAM1, IL8, NFKBIA, PTGS2, TNF*
EBV LMP1 signaling	*CCL20, IL8, NFKBIA, TNF*
Type II interferon signaling (IFNγ)	*CXCL10, GBP1, ICAM1, IL1B, SOCS3*
Cytokines and inflammatory response	*CXCL2, IL1A, IL1B, TNF*
Canonical NFκB pathway	*NFKBIA, TNF, TNFAIP3*
IL10 pathway	*IL1A, IL1B, SOCS3, TNF*
IL15 signaling pathways and their primary biological effects in different immune cell types	*CCL3, CCL4, TNF*
Chemokine superfamily pathway: human/mouse ligand–receptor interactions	*CCL2, CCL20, CCL3, CCL4, CXCL10, CXCL2, IL8*
Chemokine superfamily pathway: human/mouse ligand–receptor interactions	*CCL2, ICAM1, IL1B, IL8, TNF*
TNF signaling pathway	*CCL2, CCL20, CXCL10, CXCL2, ICAM1, IL1B, NFKBIA, PTGS2, SOCS3, TNF, TNFAIP3*

alanine-substituted peptides were created and purified $CD14^+$ cells were pulsed with each peptide to determine their response as measured by cytokine release. This led to the identification of five peptides, P1A8 (designation for peptide, P1, with alanine substitution of the 8th residue), P2A0, P2A5, P3A12, and P4A10 that stimulated maximal secretion of inflammatory cytokines by $CD14^+$ cells.

The effects of these peptides on a broader set of immunostimulatory cytokines, including IL12p70, MIG, and TNF, were then measured in pulsed $CD14^+$ cells (**Fig. 3A**). In all instances, significant cytokine induction was observed after treatment with each of the five peptides. To further characterize the effect of these peptides on $CD14^+$ cells, NanoString analysis was performed. Consistent with our previous observations with murine cells that CD200 exposure suppresses TNF signaling in APCs and CD200-mimic peptides reverse that effect, the human peptides, P1A8, P2A0, and P4A10, induced a notable increase in mRNA expression of cytokines associated with the TNF signaling pathway (**Table 1**). These results were recapitulated using a NanoString platform designed to detect the mRNA expression of TNF-regulated cytokines (**Fig. 3B**). The three peptides (P1A8, P2A0, and P4A10) that consistently induced potent upregulation of mRNA expression of TNF associated cytokines were-selected for subsequent analysis.

Targeting CD200ARs enhances DC differentiation

The NanoString analysis suggested that stimulation of monocytes with P1A8, P2A0, and P4A10 induced expression of many genes

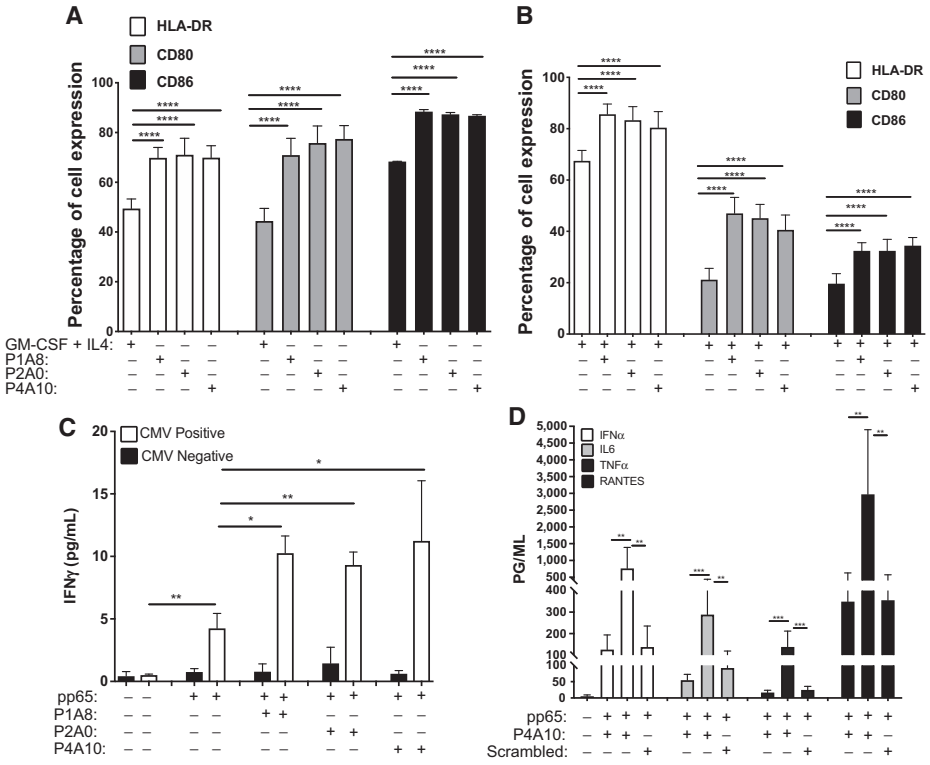

Figure 4.

Targeting CD200ARs enhances DC maturation. CD14-purified cells were pulsed with GM-CSF + IL4 or equimolar ratios of peptide P1A8, P2A0, or P4A10 (**A**) or GM-CSF + IL4 with equimolar ratios of peptide P1A8, P2A0, or P4A10 (**B**) and incubated for 48 hours. Cells were harvested and phenotyped for CD80, CD86, and HLA-DR. **C,** Immature dendritic cells were pulsed with the CMV antigen pp65 ± equimolar ratios of peptide P1A8, P2A0, or P4A10. Cells were washed and autologous T cells were added and incubated for another 48 hours. Supernatants were analyzed for IFNγ production. **D,** GM-CSF + IL4–derived iDCs were pulsed the CMV antigen pp65 ± P4A10. Cells were washed, autologous T cells were added and incubated for another 48 hours. Supernatants were analyzed for IFNγ, IL6, TNFα, and RANTE. Error bars, SD ($n = 3$ donors each run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

implicated in DC maturation (**Table 1**). To substantiate this observation, $CD14^+$ cells isolated from healthy human donors were pulsed with GM-CSF + IL4 or one of the peptides, P1A8, P2A0, or P4A10. These studies demonstrate that hCD200AR-Ls induce the differentiation of CD14 cells into iDCs. This population of cells has decreased CD14 expression and increased expression of costimulatory molecules, CD80/86 and HLA-DR, compared with cells treated with GM-CSF + IL4 ($P < 0.0001$; **Fig. 4A**). Moreover, we observed synergistic upregulation of CD80/86 and HLA-DR when $CD14^+$ cells were incubated with GM-CSF + IL4 and each of the peptides ($P < 0.0001$; **Fig. 4B**). These results show that P1A8, P2A0, and P4A10 pulsing enhances differentiation of $CD14^+$ monocytes from healthy donors into iDCs ready for antigen priming.

To assess the effects of the peptides on an antigen-specific response, GM-CSF + IL4–induced iDCs were pulsed with the CMV antigen pp65 with and without an equimolar ratio of P1A8, P2A0, or P4A10. Autologous T cells were incubated with the iDCs for 48 hours and IFNγ was measured in supernatants. pp65 exposure increased T-cell IFNγ production approximately 4-fold (**Fig. 4C**). Addition of P1A8, P2A0, or P4A10 with pp65 induced an additional two- to threefold increase in IFNγ release demonstrating that these peptides can enhance DC induction of human T-cell antigen-specific response. We tested the effect of P4A10, the most potent of the three peptides, on T-cell secretion of inflammatory cytokines after pp65 antigen presentation by dendritic cells. We observed that DCs pulsed with P4A10 induced an increase in secretion of IFNγ, IL6, TNFα, and RANTES by 5.4-, 5.6-, 16.6-, and 16.3-fold, respectively, by T cells. Pulsing with a scrambled peptide as a control failed to enhance the pp65 response (**Fig. 4D**). These results show that P4A10 enhanced the ability of APCs, specifically iDCs, to induce a T-cell–mediated immune response.

Validation of the GMP peptide

Because of issues with the stability of P4A10, the peptide that induced the most potent responses in canine, we opted to produce

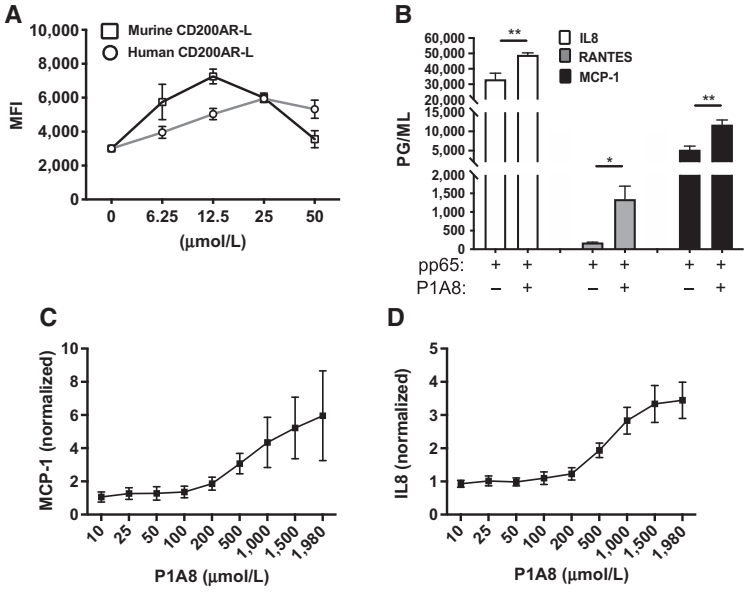

Figure 5.
Validation of the GMP peptide. **A,** HEK293 cells were pulsed with the murine P1A12 and human P1A8 fluorescently labeled CD200AR-L and analyzed for binding. **B,** GM-CSF + IL4–derived iDCs were pulsed the CMV antigen pp65 ± P1A8. Cells were washed, autologous T cells were added back, and incubated for a further 48 hours. Supernatants were analyzed for IFNγ production. $CD14^+$ cells were pulsed with different concentrations of P1A8 and analyzed for MCP-1 (**C**) and IL8 production (**D**). Cells were normalized to nonpulsed cells ($n = 3$ donors run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

P1A8 for translation into a phase I clinical trial in patients with GBM. The murine correlate of P1A8 showed the greatest efficacy in our murine survival model. To insure the GMP-grade peptide retained activity following production and formulation for vialing, we compared its binding kinetics to that of the murine peptide to HEK293 cells and showed no significant differences (**Fig. 5A**). Next, we tested the GMP peptide using a human CMV model to assess an anti-pp65 response and demonstrated that the

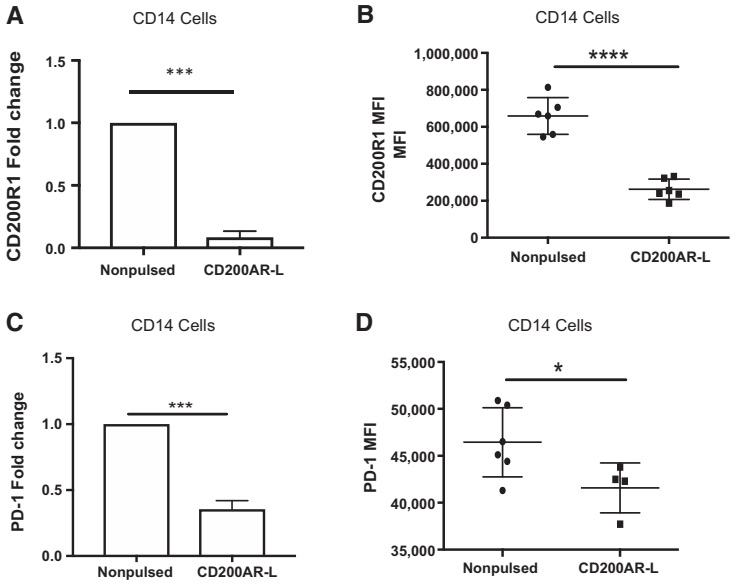

Figure 6.
Inhibitory receptor, CD200R1, is downregulated by CD200AR-L. $CD14^+$ cells were pulsed with CD200AR-L and analyzed for changes in CD200R1 transcription (**A**) and protein levels by flow cytometry (**B**). $CD14^+$ cells were pulsed with CD200AR-L and analyzed for changes in PD-1 transcription (**C**) and protein levels (**D**) by flow cytometry ($n = 3$ donors run in triplicate; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ by t test).

manufactured peptide maintained its ability to enhance an antigen-specific response (**Fig. 5B**). Finally, we assessed the binding kinetics of the GMP-grade peptide to CD14 cells and found a dose response of cytokine induction that peaked at approximately 1,500 μmol/L (**Fig. 5C** and **D**). The results from all of these experiments confirm maintenance of immunostimulatory activity of the GMP-grade peptide.

APCs primed with P1A8 downregulate the expression of CD200R1

We have demonstrated that targeting CD200ARs activates the immune system, in part, by overpowering the suppressive effects of CD200. To gain a better understanding of this mechanism, we pulsed human CD14 cells with GMP-grade P1A8. In **Fig. 6A** and **B**, we show that this treatment decreased the expression of the inhibitory receptor, CD200R1. Therefore, APCs in the draining lymph nodes or glioblastoma microenvironment that have been exposed to our peptide should be resistant to the effects of soluble CD200 from the tumor. This suppression was not observed in mock-treated control reactions. Interestingly, downregulation of PD-1 on APCs was also observed ($P = 0.005$; **Fig. 6C** and **D**). These results open the possibility of overcoming CNS immunosuppression through modulation occurring outside of the CNS.

Discussion

Immune checkpoint inhibitors are currently at the forefront of developing immunotherapies (22, 23). The most clinically successful have been those against CTL-associated protein 4 (CTLA4), programmed cell death 1 receptor (PD-1) and its ligand, programmed cell death ligand 1 (PD-L1). However, tumors can inhibit the antitumor immune response through multiple checkpoints hindering the use of these inhibitors as monotherapy. This is particularly critical for high-grade malignant brain tumors with a relatively low mutational burden and/or low immunogenicity. Therefore, multiple checkpoint inhibitors are often used concomitantly to enhance survival, but this practice frequently causes serious immune-related adverse events (6, 7).

Currently, no single FDA-approved checkpoint inhibitor has demonstrated significant efficacy in patients with high-grade glioma. Here, we explore an alternate paradigm of immune checkpoint inhibition at the site of autologous tumor vaccine inoculation outside of the CNS. Our previous studies provided compelling evidence that the CD200 immune checkpoint protein in tumor lysate suppresses the ability of APCs to trigger an effective antitumor immune response through locally recruited T cells (10, 12).

Several rigorous studies have provided evidence that targeting the CD200 checkpoint enhances immunotherapy (24–28). In the most advanced of these studies, a mAb against CD200, ALXN6000, was evaluated in a clinical trial (NCT00648739) initiated in 2008 for patients with relapsed or refractory B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma. The results of this study that was terminated before completion were recently published that showed some efficacy of ALXN6000 treatment in patients with B-CLL, but progressive disease in all patients with multiple myeloma (29).

We believe that there are problems associated with the use of an anti-CD200 antibody for GBM, some of which are exemplified by the Alexion results: (i) antibodies fail to cross the blood–brain barrier, which limits their efficacy in CNS tumors; (ii) multiple cells including neurons and immune cells express CD200 (30, 31); therefore, the use of an anti-CD200 antibody might cause off-target toxicity and decrease effectiveness. Alexion reported that in patients with B-CLL, 95% of the patients with B-CLL had as much as a 98% reduction of $CD200^{+}CD4^{+}$ T cells. The loss of these immune cells may create an immunocompromised condition and may be the reason that only one achieved a durable partial response while most of the patients had stable disease. Although they reported CD200 expression on B-CLL cells was greatly reduced in 67% of patients, CD200 is secreted from tumors (32) and this parameter does not correlate with tumor reduction.

We chose to develop peptide ligands to target the CD200-activating receptors on APCs. Peptides have the ability to penetrate further into tissue (33) and have higher activity per unit mass, greater stability, and reduced potential for nonspecific binding that may result in decreased toxicity (34). Despite having clearly demonstrated clinical efficacy of a synthetic peptide ligand, the mechanism that modulated an immune response through activation receptor binding was unknown. We developed three murine CD200AR-Ls that demonstrated different survival rates in a murine glioma model. The murine ligand, P1, that was predicted to bind primarily to CD200AR4 enhanced survival in our murine glioma model, whereas other ligands predicted to bind primarily to CD200AR2 and CD200AR3 enhanced survival in our murine breast tumor model, but had no efficacy in the glioma model (12). Because our interest was primarily in the role of activation receptors (CD200ARs), we focused cell lines expressing CD200AR2–4. We observed that cells expressing certain CD200AR combinations, specifically 2&3 and 3&4, responded to stimulation by the P1 ligand. In contrast, cells expressing other receptor combinations, including 1, 2, and 3, 1, 3, and 4, or 2, 3, and 4, failed to bind P1 and had no increase TNFα production, and the CD200AR2&4 cell line bound P1, but had no concomitant increase TNFα production. We suggest that ligation of the various ARs produces different immune responses, a phenomenon that is currently under investigation in our laboratory. These data lead to the hypothesis that the activation receptors (CD200ARs) function as complexes to modulate immune activation. This could explain our observation that targeting different CD200ARs induced different survival benefits in our breast carcinoma and glioma murine models (12).

Our studies provide compelling data that the presence of the CD200 protein in brain tumor lysates suppresses the capacity of local APCs to activate recruited T cells and trigger an effective antitumor immune response (10, 12). We built on this earlier observation and tested peptides in an attempt to target CD200-mediated immunosuppression and successfully reversed the immunosuppressive effect of CD200 in murine studies.

While murine brain tumor models have yielded valuable insights into the etiology of glioblastoma, the vast majority of novel therapies that showed enormous promise in these models subsequently failed in clinical studies. Recent attention has been focused on companion dogs as a translational model due to their strong anatomic and physiologic similarities to humans and the sheer number of pet dogs that are diagnosed and managed with cancer each year (35–37). Strong similarities have been shown between the canine and human genome, especially with respect to gene families associated with cancer. These combined factors suggest cancer in companion dogs as a viable model for preclinical human cancer research including brain tumors (38–40). Because of our success in the canine CD200 trial (41), the human CD200AR-L, P4A10, most analogous to the canine CD200AR-L, was initially selected for a human phase I trial. However, the charges within this peptide made it difficult to scale up for GMP production, therefore

we chose P1A8, analogue of the murine peptide that provided the greatest survival benefit in our murine studies. Our human *in vitro* studies demonstrated that hP1A8 enhanced DC differentiation, maturation, and cytokine production, as well as an antigen-specific T-cell response. In addition to cell activation, we also demonstrated downregulation of the inhibitory receptor, CD200R1. This is important because CD200 secreted by the tumor suppresses the ability to mount an antitumor response due to binding of CD200/CD200R1 on immune cells and CD200 is upregulated in the tumor-associated vascular endothelium (as evidenced in our earlier studies; ref. 10) limiting the ability of immune cells to extravasate into the tumor microenvironment in response to immunotherapy. We believe downregulation of CD200R1 will allow immune cells to move into the tumor microenvironment from the tumor vasculature. Moreover, the downregulation of both CD200R1 and PD-L1 should render immune cells resistant to tumor-induced suppression in the tumor microenvironment. We hypothesize that the significant survival response seen in the canine preclinical trial is due to the ability of the CD200AR-L peptide to override the suppressive effects of multiple immune checkpoints.

Disclosure of Potential Conflicts of Interest

G.E. Pluhar is an advisory board member/unpaid consultant for OX2 Therapeutics. D.A. Largaespada is an employee/paid consultant for Surrogen, Inc., reports receiving commercial research grants from Genentech, and holds ownership interest (including patents) in NeoClone Biotechnology, ImmuSoft, formerly Discovery Genomics, Inc., and Recombinetics, Inc. C.L. Moertel and M.R. Olin are employees/paid consultants for and hold ownership interest (including patents) in OX2 Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Writing, review, and/or revision of the manuscript: E. Ampudia Mesias, G.E. Pluhar, S.K. Rathe, D.A. Largaespada, Y.Y. Sham, C.L. Moertel, M.R. Olin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.L. Moertel, M.R. Olin

Study supervision: G.E. Pluhar, Y.Y. Sham, C.L. Moertel, M.R. Olin

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